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IMMUNOLOGIC ASPECTS OF PARASITIC INFECTIONS

PAN AMERICAN HEALTH ORGANIZATION
Pan American Sanitary Bureau, Regional Office of the
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IMMUNOLOGIC ASPECTS OF PARASITIC INFECTIONS

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NOTE

At each meeting of the Pan American Health Organization Advisory Committee on Medical Research, a special one-day session is held on a topic chosen by the Committee as being of particular interest. At the Sixth Meeting, which convened in June 1967 in Washington, D.C., the session reviewed current information on the immunology of parasitic infections and brought out facets of the subject about which more knowledge is needed. This volume records the papers presented and the ensuing discussions.

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OPENING STATEMENT

Otto Bier, Moderator

When the Advisory Committee on Medical Research decided last year that during this meeting a special session should be dedicated to the immunologic aspects of parasitic infection, our Chairman, Professor René Dubos, rightly insisted upon a very important point. The session was not intended to cover the whole field of parasitic immunity, but rather should concentrate on a few selected topics in which discussion could eventually lead to new lines of investigation based, whenever possible, on simplified experimental models.

As one of the members of the committee that suggested the theme, I was inevitably charged with the primary responsibility of organizing the session and acting as moderator. Knowing practically nothing about parasitology, and having no experience at all in the field of immunoparasitology, I was thus put in the awkward position of having to fulfill a job for which I am really not prepared. Unlike Monsieur Jourdain, who could easily be convinced "qu'il faisait de la prose sans le savoir," I am not at all convinced, in spite of having dedicated thirty years of my life to research on immunology, that I shall be able to act as a competent moderator in this session.

However, the decision had been made, and we had to go ahead. Just after the meeting last year, a prolonged discussion was held in Geneva with Drs. Niels K. Jerne, Howard C. Goodman, Zdenek Trnka, and Maurício Martins da Silva. Our conclusion was rather pessimistic—we left with the fear that our session could result in an inferior duplication of the excellent meeting on immunology and parasitic disease that had been convened by WHO not long before in Ibadan, Nigeria, with the participation of a most competent group of experts in basic and parasitic immunology.

In spite of this, a program was outlined and Dr. Martins da Silva was given the task of preparing the first draft through further consultations with "immunologically competent" people in the United States. With the expert advice of Drs. Louis Olivier, E. J. L. Soulsby, P. P. Weinstein, and Frans C. Goble, a very fine draft was prepared within a relatively short time. Most important of all, Dr. Martins da Silva succeeded in getting the collaboration of competent immunoparasitolgists and basic immunologists not only for the presentation of the topics but also for their discussion.

In accordance with the requirements laid down by our Chairman and other members of the committee, the session has been organized in such a way as to emphasize the fact that its objective is to encourage better work on

mechanisms or mechanics of immunity in parasitic infection by bringing out aspects of the topics about which more knowledge is needed and opening new avenues of approach. It was also agreed that, in view of the prime importance of schistosomiasis and Chagas' disease on the American continent, these parasitoses should be used as illustrative examples whenever possible.

The seven topics selected will be presented in a logical sequence: parasite antigens and antibodies, cellular reactions, and effects of the immune response on both the parasite and the host. Before we start with the topics related to parasite antigens and their antibodies, we shall have an introduction by Dr. William H. Taliaferro. Nobody could be better qualified than Dr. Taliaferro to give this introduction. Besides being a pioneer in the field of parasitic immunology—in which his contributions are massive and frequently fundamental—he is also outstanding in the field of basic immunology by virtue of his important work during the past decade, in collaboration with his wife, Dr. Jaroslow, and others, on the effect of X-radiation on antibody formation.

A RETROSPECTIVE LOOK AT THE IMMUNOLOGIC ASPECTS OF PARASITIC INFECTIONS*

William H. Taliaferro

My function today, as I understand it, is to emphasize the importance of some of the earlier basic research on the immunology of parasitic infections as a foundation for the very interesting papers that follow.

Each of us has his own ideas on basic as compared to applied research, even though we realize that there has always been a two-way street between them and that they supplement each other. In 1948 I defined a basic scientist as one who approaches his research in terms of individual interest to a greater extent than the applied scientist and who is largely dependent for a successful outcome upon lucky guesses, inspiration, or—to use a fashionable word—serendipity (103). The basic scientist is interested primarily in how nature works, while the applied scientist is interested primarily in benefiting mankind. At times, however, both aims may be accomplished.

Chance played a remarkable role in my research. When Dr. Robert Hegner invited me to join his Department of Protozoology at Johns Hopkins University in 1919, I had seen only one parasitic protozoon—it happened to be *Trypanosoma lewisi*—and I had had only a basic training in general physiology. That background, combined with a knowledge of the genetic studies of Dr. Herbert Jennings on variations in free-living protozoa, started me off on my work on *T. lewisi*. This sketchy beginning resulted in a study of the rat-*T. lewisi* relationship that has continued to intrigue me ever since (93, 96, 98, 99, 109, 113, 118). Moreover, I feel particularly fortunate in having my co-worker Philip D'Alesandro (22) investigating the specific factors involved in the mechanism of ablastic action on *T. lewisi*.

In 1955 I congratulated the investigators in tropical medicine for the healthy respect with which they regarded both basic and applied biological research, especially in view of the tremendous advances in applied science during and after World War II (107). This attitude stimulated and benefited both fields and led to the rapid application of many fundamental findings to chemotherapy, control procedures, and allied problems. This statement is dramatically substantiated by the intensive work of Jarrett and his co-workers, at the Veterinary School of the University of Glasgow, on bronchitis in cattle caused by the lungworm, *Dictyocaulus viviparus*, which has led to the large-scale production of a vaccine consisting of heavily irradiated larvae (73). Similar work on other infections has already been started, but successful immunizations have not yet been reported. This field will undoubtedly be vigorously attacked, but the timing and dosage of irradiation and the time of administering the challenging antigen will obviously have to be carefully standardized and evaluated, as has already been found necessary in studying the hemolysin response in rabbits (see later discussion).

I also pointed out at that time that the defi-

* Work supported by the Atomic Energy Commission.

nition of what is fundamental can only be relative because what is fundamental for the clinician and public health worker may be applied for the biologist and what is fundamental for the biologist may be applied for the chemist or physicist. Thus, as biology develops, we move toward the physical sciences. This situation is especially evident today, when biology is being carried to the molecular level by the accelerated use of tools from the fields of chemistry, physics, and mathematics. Biologists are now working with electron microscopes, with refined chemical analyses and with complex methods for determining atomic and molecular structure. At the other end of the spectrum, students are beginning to graduate in the field of biological engineering. With biology being approached at these various levels, the tenuous line between basic and applied work is being erased, but I predict that the "lone wolf," the young person with an unorthodox mind and with apparently improbable ideas, will uncover disproportionately outstanding results.

To turn now to the basic immunological work on parasites, it seems remarkable in retrospect how disinclined some investigators, especially helminthologists, were in the first quarter of this century to recognize that acquired immunity develops against the animal parasites (20, 21, 95, 100, 102). What makes it all the more surprising is that the classic studies on trypanosomes by Ehrlich (29, 30) were reported in 1907 and that the equally valuable studies on immunity in malaria were begun in 1910 by the Sergents (80). The main reason for this situation was that most parasitologists were involved in systematics and life histories, while the medical research workers were concerned with diagnosis, symptomatology, pathology, and therapeutics. Immunology with respect to animal parasites was in its infancy. Knowledge was scarce and hit-or-miss—usually fragmentary for a given host-parasite relationship and often nonexistent.

Fortunately, the results of the study of some host-protozoan relationships were sufficiently clarified by 1926 to allow Hegner (39) to state that host-parasite relationships in the trypanosomiases and malarias were being aligned with immunology of bacterial infections. By 1929, a series of papers demonstrating the production of antibodies to animal parasites had been published and, in that year, my book *The Immunology of Parasitic Infections* (95) appeared. There I pointed out the uniqueness of parasites in that their large size and accessibility allowed them to be followed *in vivo* in relation to the host's reactions and to be collected in large quantities for the preparation of antigens for *in vitro* analysis.

During the succeeding 30 years, immunological phases of parasitic infections were attacked with increasing interest (21, 90, 97, 100, 101, 102, 105, 108). More recent reviews, such as those by Garnham and others (33, 40, 45, 57, 70, 78), will undoubtedly be mentioned by subsequent speakers. In most of this work, acquired antibody-mediated mechanisms were reported to be superimposed upon innate, nonspecific, heterogeneous mechanisms that limit invasion or growth of the parasites after invasion. Many of the innate mechanisms are inherited and in an over-all sense are more important than acquired immunity (33).

In the remainder of this paper I shall describe some results obtained by three different experimental approaches, which possess inherent advantages. These are the cellular phases of immunity, the separation of parasiticidal from reproduction-inhibiting activities, and the role of immunity in a well-known antigen-antibody system.

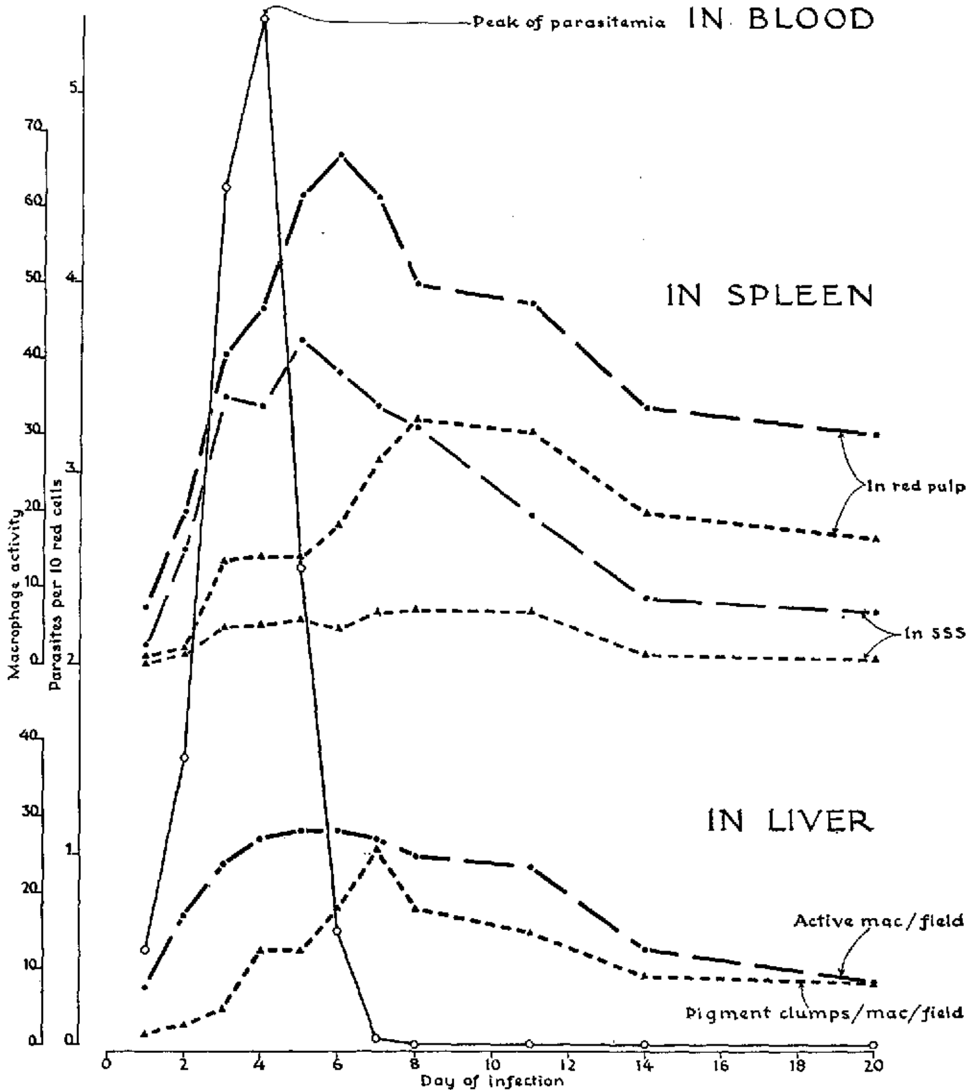
The cellular phases of immunity

Various malarias have been invaluable in studying the cellular phases of immunity (101, 102, 105), especially because malarial pigment serves as a marker for a considerable time after the parasite has been digested. From 1931 to 1937, with my colleagues Paul Cannon (14, 112), William Bloom (7, 111), and Hugh Mulligan (115), I studied the increase in macrophages in canaries and monkeys as they phagocytosed plasmodia and overcame infections. Hematological studies were also carried out with C. Klüver (114). One thing became

apparent from this work: our data did not support the idea, current at that time, that additional phagocytes needed for any but the mildest infections arose exclusively by the division of pre-existing histogenous macrophages in the affected area. We concluded that additional phagocytes arose chiefly through the mitotic division of lymphocytes and monocytes in hematopoietic tissues and their migration via the blood into strategic tissues and organs where they subsequently developed heteroplastically into macrophages. For purposes of the following brief discussion, I shall use the term "lymphocyte" to include lymphocytes of all sizes (small, medium, and large) and restrict the term "monocyte" to the typical blood monocyte, which is closely related to the lymphocyte.

To show some of the changes found, I have selected two figures taken from later work

FIGURE 1. The parasitemia of blood-induced *Plasmodium lophurae* (unbroken line), in chickens initially infected, and macrophage activity of the host (dash lines), as gauged by macrophage content of malarial debris (active mac) and pigment clumps, in the red and white (SSS) pulp of the spleen and in the liver. From Taliaferro and Taliaferro (126) by permission of the authors and the University of Chicago Press.



(126) on chickens infected with *Plasmodium lophurae.* In this severe but nonlethal infection, macrophages increased (Figure 1); lymphocytes, after being depleted, also increased (Figure 2). After the injection of a large number of plasmodia, the parasite count rose to a peak of 5.5 parasites per 10 red cells on day 4 and subsided to a subpatent level by day 8. As gauged by the number of macrophages containing malarial material per microscopic field, phagocytic activity was low on day 1 in both the spleen and liver, but reached peaks just after the parasitemia peak and subsided gradually thereafter. Figure 1 emphasizes the mobilization of phagocytic macrophages that suppress the infection.

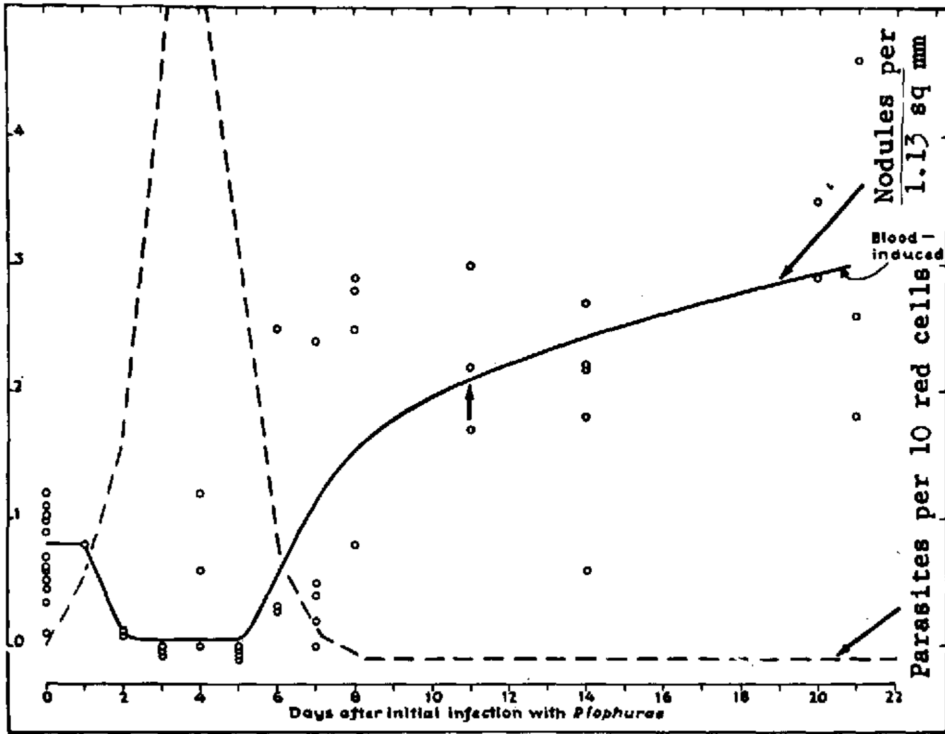
Additional macrophages, in our opinion, were supplied by lymphocytes as gauged by changes in the lymphatic nodules of the spleen (Figure 2). The nodules showed a mean number of 0.8 per microscopic field before infection. They rapidly disappeared in 2 days, remained depleted through day 5, reappeared on day 6, reached a level of about 2 per field on day 11, and attained an approximate 3.5-fold increase over normal on day 22. The reappearing nodules frequently contained 10 to 20 dividing lymphocytes per section and were often abnormally large. In contrast, reticular cells lining the sinusoids and macrophages throughout the spleen and other tissues rarely divided. During the following month and a half, the nodules gradually declined in number and size until they reached the normal level at three months. During the depletion and subsequent increase of lymphocytes, inflammatory mononuclear cells were numerous in the spleen and in other strategic tissues. These cells, also identified as mononuclear exudate cells or polyblasts, varied in size and appearance over a wide range as the cytoplasm swelled and the chromatin in the nuclei became less compact. They were best seen in thinly cut, well-stained sections of tissues fixed immediately upon the death of the infected host.

Thus, we concluded that the additional macrophages needed to suppress the malarial infection were supplied by the division and heteroplastic transformation of lymphocytes. This idea is embodied in the term "lymphoid-macrophage system," which Mulligan and I (115) proposed in 1937, in preference to the term "reticulo-endothelial system," which was advanced by Aschoff (2) in 1927 to embrace all cells involved in defense but which did not include lymphocytes, monocytes, or intermediate polyblasts. A fuller account of this subject may be found in an earlier publication (106).

Our results fell in line with the classic work of Maximow beginning in 1902 (58, 60-62). He found that cells from the blood began to migrate early and continued to migrate into an inflamed tissue. In the tissue, the mononuclear exudate cells rapidly developed through polyblast stages into macrophages that were indistinguishable from the large tissue macrophages at 36 to 48 hours. In 1928, Bloom (6) demonstrated the transformation of lymphocytes from rabbit thoracic duct into macrophages in tissue culture.

Lymphocytes and, to a more limited extent, monocytes are a part of the mesenchymal reserves. That is, they are free, normally circulating connective tissue cells that retain to varying degrees the power to undergo heteroplastic development into more specialized cell types (59, 106). These reserves are on occasion sources of red cells, granular leukocytes, phagocytes, and other cells of the connective tissue involved in mechanical support and repair of injuries of certain types. An instance of their critical importance in the adult organism is illustrated in Figure 3, which shows the parasitemia and the number of lymphatic nodules during a superinfection by *P. lophurae* of chickens about 1.5 months after initial infection. The parasite count rose to a peak of 3 parasites per 10 red cells on day 3 and subsided to a subpatent level by day 5. Just before superinfection, the number of nodules was high, with a mean of 2.4 per field, because of residual activities connected with the initial infection; they decreased rapidly for 2 days, increased to a peak of about 2 per field on day 11, and then declined slowly. As compared to the initial infection (Figure 2), the higher

FIGURE 2. The parasitemia of blood-induced *P. lophurae* (dash lines), in chickens initially infected, and the number of lymphatic nodules (data points and unbroken line). As the parasitemia increased, the nodules decreased in number: then, as the parasitemia declined and reached a subpatent level, the nodules markedly increased. Modified from Taliaferro and Taliaferro (126) by permission of the authors and the University of Chicago Press.



level of nodules at the beginning of the superinfection, which represented augmented mesenchymal reserves, was followed by a milder parasitemia and an earlier decline in the number of nodules after the superinfection was suppressed.

To illustrate the rapid activity of blood leukocytes, I have selected some unpublished camera lucida drawings from the skin of rabbits before and immediately after the subcutaneous injection of a few *Trichinella* larvae (Figure 4).* These larvae serve as markers of the cellular activities, as did the malarial pigment. The first few hours after introduction of the larvae are important because of leukocytic migration and development.

Cells of the normal tissue consisted chiefly of faintly staining fibroblasts; macrophages and their close relative, adventitial cells; and endothelial cells lining the sinuses. Blood leukocytes were rare: in Figure 4A, only one is seen. Half an hour later (Figure 4B), this picture had markedly changed in the vicinity of the larvae. Numerous leukocytes were migrating from the venule into the tissue and around the larvae. They consisted of heterophils (polymorphonuclears), eosinophils, and lymphocytes, all of which were normal in appearance and unchanged in size. At six hours, the site around the larvae was filled with leukocytes. The small area in Figure 4C shows 80 heterophils, 5 eosinophils, and 24 mononuclear exudate cells. The latter are marked by arrows and are variously labeled "lymphocytes," "monocytes," "monocytoid lymphocytes," and "medium polyblasts." They ranged in size and appearance from the small lymphocyte in the upper left, through monocytes or monocytoid lymphocytes, to the medium-sized polyblast shown a

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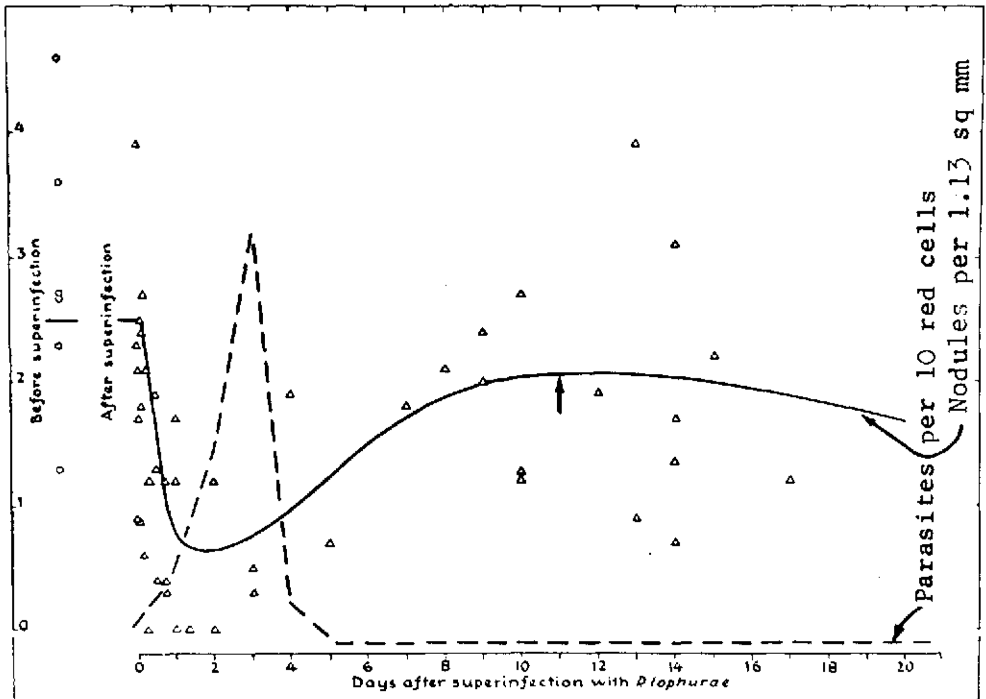
* I am indebted to Mrs. E. Bohlman Patterson for these drawings and for those in Figures 5 and 6.

FIGURE 3. The parasitemia of blood-induced *P. lophurae* (dash lines) in chickens during a superinfection and the number of lymphatic nodules (data points and unbroken line). The nodules decreased markedly in number as the parasitemia increased: then they rose to a peak on day 11 and declined. These changes were superimposed on a high base line because of the residual activation of the initial infection (see Fig. 7). Modified from Taliaferro and Taliaferro (126) by permission of the authors and the University of Chicago Press.



little below the lymphocyte. At this time, hypertrophy in the inflammatory mononuclear cells, as gauged by gradual swelling of the cytoplasm and lightening of the nuclear chromatin, was evident but not pronounced. At 18 hours, the small area in Figure 4D contains 35 heterophils (many of which were degenerating), 3 eosinophils, and 18 inflammatory mononuclear cells. The latter ranged in size from the small migrating lymphocyte seen at the bottom right, through polyblasts, to the large hematogenous cells seen at the top left. Some of these larger cells were phagocytic and were approaching in appearance the large tissue macrophage shown at the top right. Phagocytosed heterophil remnants are readily seen in the active macrophages. Fibroblasts were inactive throughout, and no dividing cell of any kind

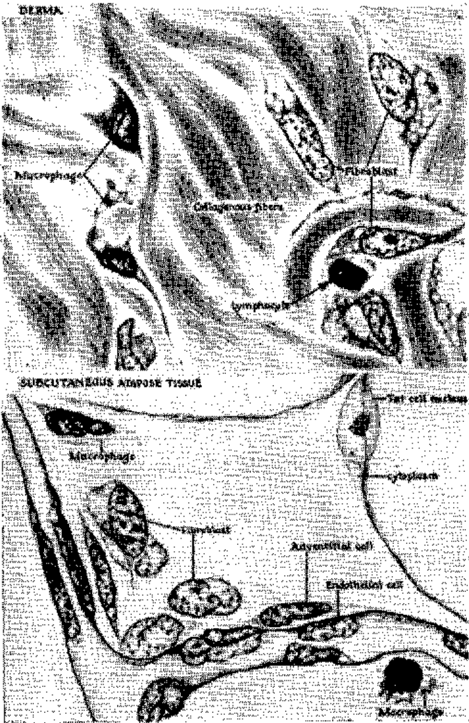
FIGURE 4 (opposite). Camera lucida drawings from normal skin and from skin near *Trichinella* larvae ½, 6 or 18 hours after the larvae were injected intracutaneously into rabbits. The tissues were fixed in Zenker-formol, embedded in celloidin, and stained with hematoxylin-eosin azure II (10). The mononuclear exudate cells are identified by arrows. X 1500.

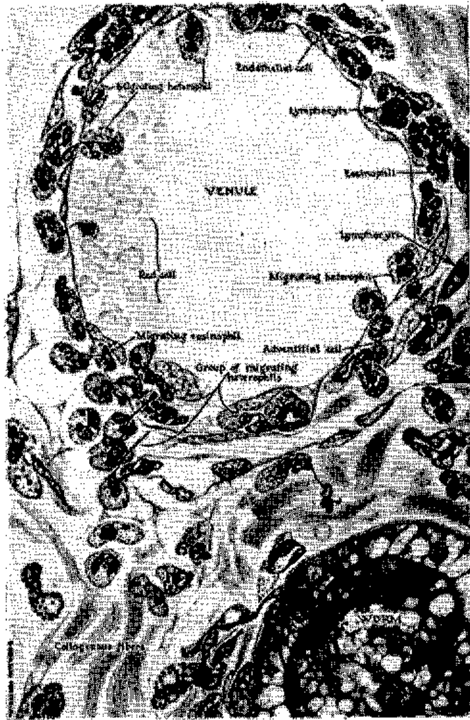
A. Normal derma and subcutaneous tissue containing inactive histogenous macrophages, adventitial cells, fibroblasts, and part of a small venule.

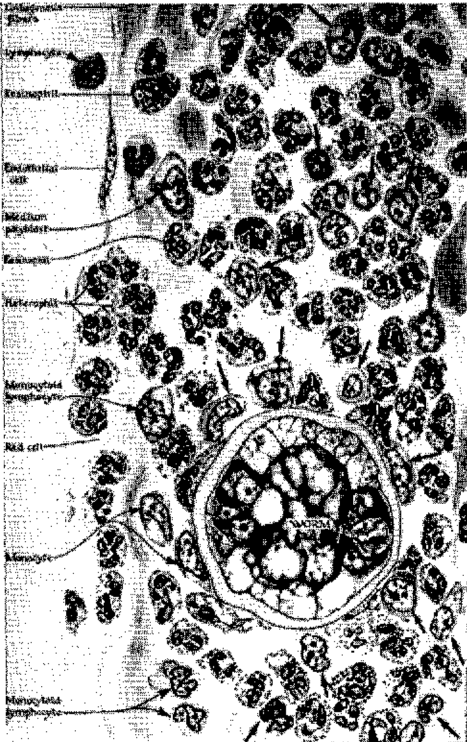
B. Small venule after ½ hour, from which leucocytes are migrating into the derma near a worm.

C. Derma after 6 hours containing a worm surrounded by leukocytes. The heterophils and eosinophils are normal in appearance and size; the mononuclear exudate cells show gradations in nuclear and cytoplasmic structures from lymphocytes through monocytoid stages to medium sized polyblasts.

D. Derma after 18 hours near a larva showing many heterophils (some are degenerating), a few eosinophils, and the heteroplastic development of many mononuclear exudate cells from a typical lymphocyte through polyblasts of medium size to large actively phagocytic hematogenous macrophages which are almost the size of the phagocytically active tissue macrophage. The phagocytosed material consists largely of remnants of leukocytes. The fibroblasts are essentially unchanged.







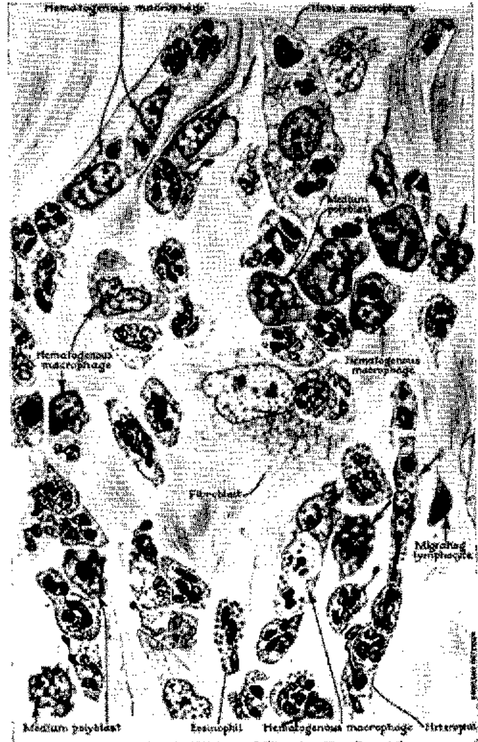


FIGURE 4

was seen in the inflamed area during all this activity. The migration of cells and the heteroplastic development of hematogenous mononuclear cells into macrophages continued for a long time thereafter.

The same rapid activity of blood leukocytes occurred in the skin of guinea pigs immediately after the intra- and subcutaneous injection of a few killed staphylococci. Figure 5 shows some unpublished camera lucida drawings of derma before and 1, 2, 6, 18, and 36 hours after the introduction of the bacteria. The only marked difference in defense against staphylococci, as contrasted with defense against *Trichinella*, was that heterophils played an early phagocytic role (Figure 5D). In other respects, the sequence was essentially similar. The leukocytes of the blood promptly migrated into the tissue (Figures 5B and 5C), and the lymphocytes and monocytes developed heteroplastically through inflammatory mononuclear stages (Figures 5D and 5E) into phagocytic macrophages. At 36 hours, heterophils and even engorged macrophages were degenerating, while tissue and hematogenous macrophages were actively phagocytosing bacteria and debris (Figure 5F). Throughout, fibroblasts were strikingly inactive, and no dividing cell of any kind was seen in the affected area. At 7 days, the cellular content of the inflamed area consisted of macrophages, a few of which were still phagocytic, fibroblasts, cells intermediate between macrophages and fibroblasts, and a few inflammatory mononuclear cells. The area contained at least twice as many cells as before the entrance of the bacteria.

Cellular reactions were also studied by Pizzi and me (117) during infections and superinfections of C_3H mice with a reticulotropic strain of *Trypanosoma cruzi* that localizes and thrives for a time in macrophages and reticular, adventitial, and Kupffer cells (Figure 6). The mice usually died in 9 to 11 days. In marked contrast, mice immunized with avirulent trypanosomes, when challenged with the virulent strain subcutaneously, were able to suppress the infection to a low level in 2 days. Histopathological material revealed that destructive processes predominated in the nonimmune mice, whereas marked myeloid, macrophage, and lymphoid proliferations protected the immune mice.

The question arises, How was the protection brought about? Antibodies were probably important, as the successful immunization suggests (71), but, in addition, stretch preparations of the subcutaneous loose connective tissue at or near the site of the challenging inoculation revealed that the free inflammatory, newly developing macrophages appeared to dispose of the parasites more effectively than the histogenous macrophages of the area, as is shown in Figure 6B. This finding needs further study —not only of the macrophage itself but also of the parasite. For example, it may be feasible to adapt some of the approaches already used to study interactions between heterophils and bacteria (89). Indeed, it would be of great interest to ascertain whether the metabolic pathways in the macrophages of normal

FIGURE 5. Camera lucida drawings from normal skin and from skin at 1, 2, 6, 18, and 36 hours near or at the site of the intracutaneous injection of killed *Staphylococcus aureus* into guinea pigs. The tissues were prepared as for Figure 4. The mononuclear exudate cells are identified by arrows. X 1500.

A. Normal derma containing inactive histogenous macrophages, fibroblasts, and part of a small venule.

B. Small venule near the bacteria after 1 hour, containing a concentration of leukocytes some of which are migrating into the derma.

C. Derma near the bacteria after 2 hours, containing a fibroblast, a phagocytic histogenous macrophage, and heterophils and mononuclear exudate cells which have migrated from adjacent venules. At the bottom is shown the phagocytic activity of the histogenous macrophages in the area at this time.

D. Derma after 6 hours, showing (1) an area near the bacteria and (2) an area containing the bacteria, which are being phagocytosed by the heterophils. Both areas exhibit a concentration of hematogenous leukocytes and some of the mononuclear exudate cells are larger than after 2 hours.

E. The derma after 18 hours, showing many medium-sized polyblasts, some of which are phagocytic. The fibroblasts are not phagocytic.

F. The derma after 36 hours, showing degenerate heterophils and phagocytes of both blood and tissue origin. The phagocytosed material consists of remnants of staphylococci and of leukocytes.

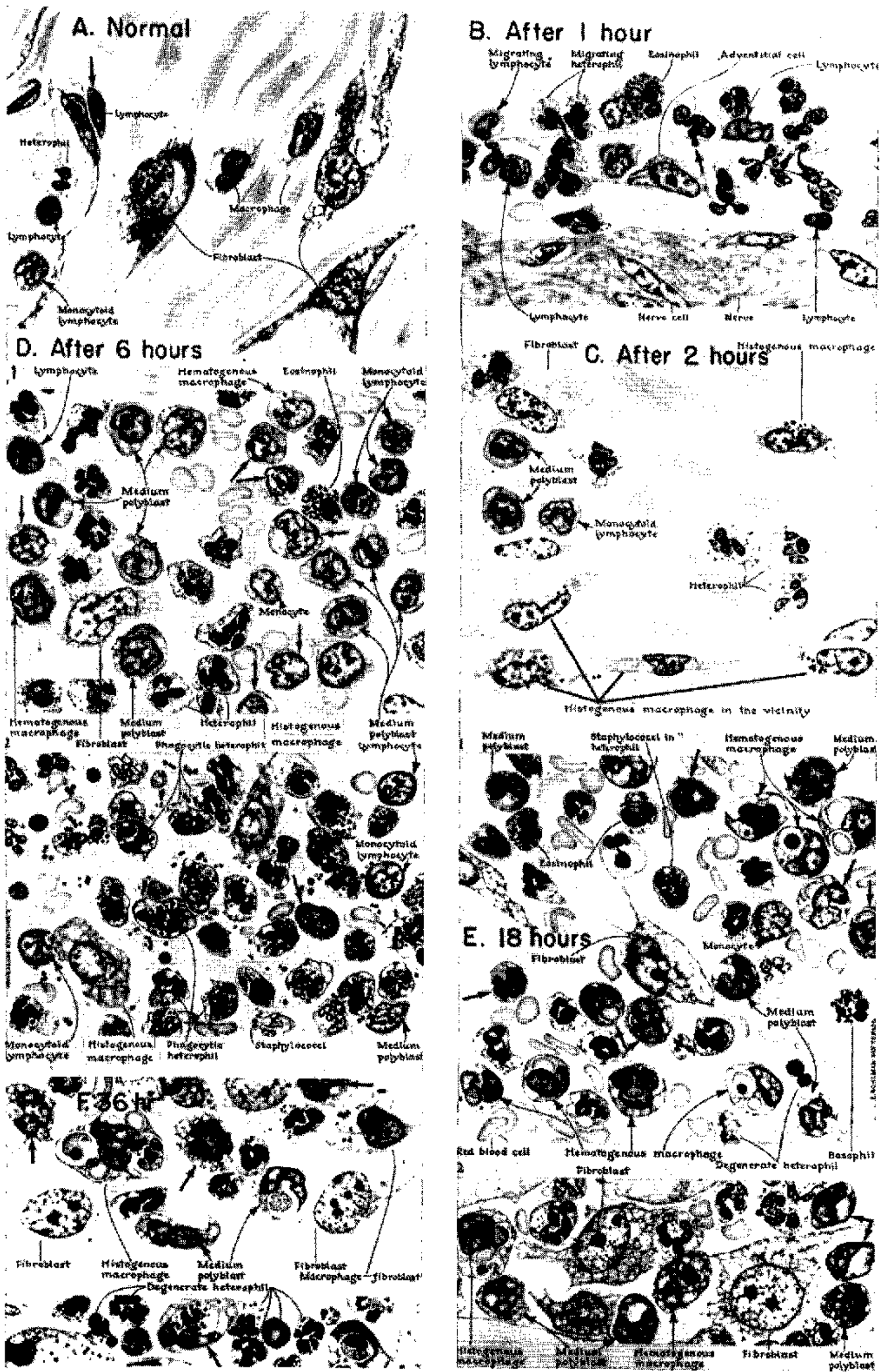
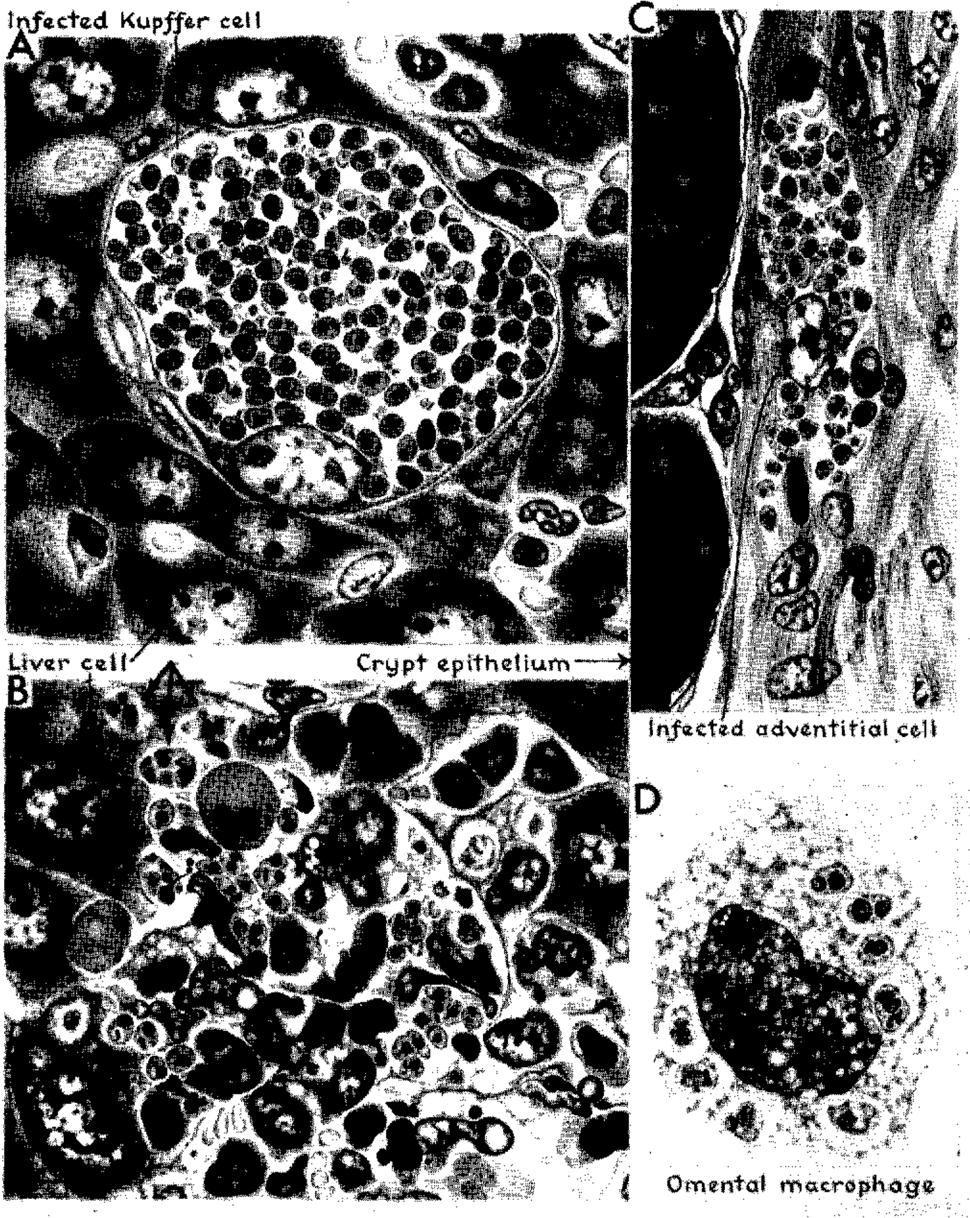


FIGURE 5

FIGURE 6. Camera lucida drawings from tissues of C_3H mice initially infected from 4 to 11 days with a virulent strain of *Trypanosoma cruzi.* The tissues were prepared as for Fig. 4 except for Fig. 6D which was stretched, dried in air, and stained with Giemsa. X 1500.

A, C, and D. Normal-appearing leishmanial stages in a Kupffer cell of the liver, an adventitial cell in the intestine, and a macrophage in the omentum, respectively.

B. Normal and abnormal (identified by arrows) parasites, which probably arose from the rupture of a cell similar to that in Figure 6A, are being phagocytosed by inflammatory macrophages (polyblasts). From Taliaferro and Pizzi (117) by permission of the authors and the University of Chicago Press.



hosts differ from those in hosts during innate and acquired immunity. Chemotaxis, enzymes, and opsonins undoubtedly play a role. Moulder and I (65) have already found that enlarged spleens in chicken malaria involve an actual increase in functional tissue and that new cells

have the same glucose metabolism as cells in the uninfected spleen.

Cellular activities of the host against other parasites have been documented—by Singer (82) with *Plasmodium berghei* in mice and by Barnett with *Theileria parva* in cattle (4), for example.

The foregoing studies, based on closely spaced, early, serial sampling of fixed tissues, strongly suggest that defense in a wide assortment of hosts against a wide assortment of parasites depends upon the mesenchymal potencies of the lymphocyte and monocyte. This concept has been maintained for decades by such workers as Metchnikoff (63), Dominici (24, 25), Maximow (58-62), Downey and Weidenreich (26, 27), Bloom (6), Jordan (51), Kolouch (56) and Rebuck (74-76), as well as by my associates and me (112, 115, 114, 106, 82, 108, 117, 126, 4). Many scientists, however, have questioned this idea (74, 137). They have even been skeptical about whether the lymphocyte, especially the small lymphocyte, has any mesenchymal potencies. This attitude is beginning to change (38, 137) as specialists use new techniques and materials. These include refined methods of tissue culture, the "skin window" technique devised by Rebuck, the intraperitoneal diffusion chamber, fluorescent dyes, radioactive antigens, autoradiography, and electron microscopy. For example, Howard *et al.* (41, 42), using genetic markers to identify cells and the graft-versus-host reaction as well as irradiation, have demonstrated that thoracic duct lymphocytes in mice, after settling in the liver, divide and acquire the character of mononuclear phagocytes, indistinguishable from the macrophages of the area. Lymphocytes have also been studied both *in vivo* and *in vitro* with regard to plasma cell and antibody formation (3, 16, 19, 67, 68, 85, 138, 139).

Thus, from a cell that 30 years ago was widely thought to have no particular function, the lymphocyte has become one that is being closely scrutinized to assess its function in defense (137).

The separation of parasiticidal and reproduction-inhibiting activities

Parasites lend themselves admirably to a study of the cellular activities of the host because of attributes such as size or pigment that act as markers for their presence in the host. These same attributes, however, hamper the study of humoral activities because large size is accompanied by complexity. Further troubles occur. The parasites reproduce, and they consist of a baffling array of antigens, some of which are certainly common to the host. Finally, the antibodies that arise are just beginning to be accurately measured (15, 52, 53). Nevertheless, antibodies have been known to exist against parasites since the detailed studies of Ritz (77) in 1914 on trypanosomes. In addition, aside from many studies on immunity to superinfection (20, 21, 95, 100, 102), Coggeshall and Kumm in 1937 (17) first established the fact that immune serum protects monkeys against lethal infections of *Plasmodium knowlesi.* They survived and their parasitemia was almost completely suppressed when they were injected initially with a suitable number of parasites and several comparatively large daily doses of immune serum from chronic drug-treated infections.

Further study fortunately revealed that parasiticidal mechanisms can be differentiated from reproduction-inhibiting activities in synchronously reproducing, blood-inhabiting species of plasmodia because the reproduction of the plasmodia and the number that die can be independently ascertained. In the absence of an adequate test for antibody, antibody-induced acquired immunity was assumed to be superimposed upon innate immunity when the parasitemia in an infection rapidly decreased after a peak, as is shown in Figure 1. Figure 7 illustrates the results of such a study for 6 malarial species (104, 120).

On the one hand, reproduction of the parasites (merozoites-produced) was progressively inhibited in four infections (Figures 7B, C, E, and F) during innate immunity and was temporarily inhibited in all six infections just after

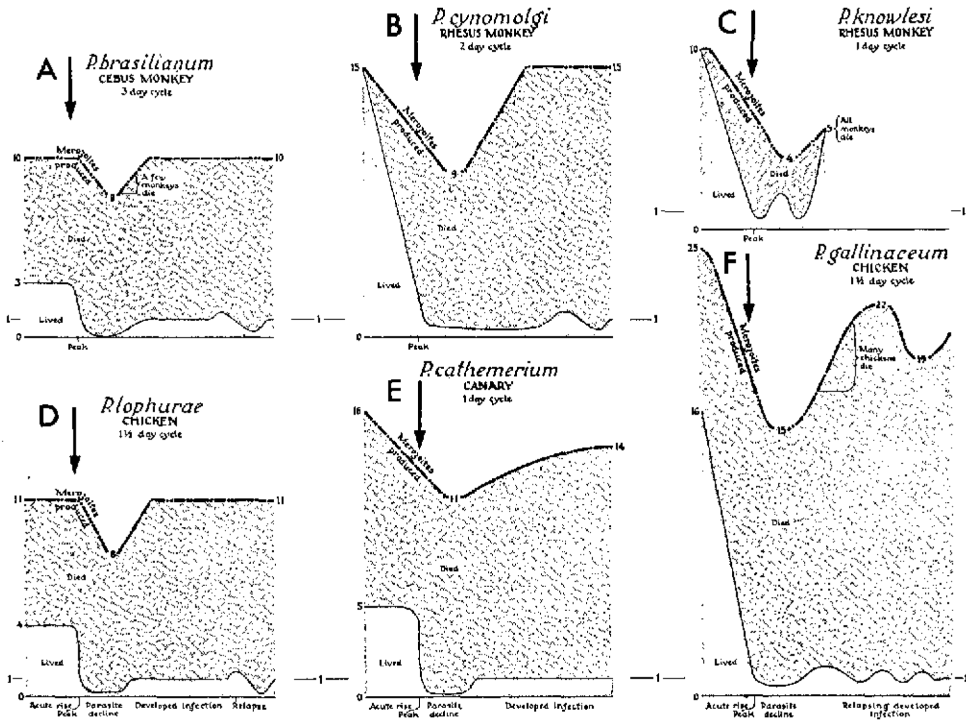


FIGURE 7. Schematic diagrams of 6 species of plasmodia during blood-induced infections of monkeys or birds showing (1) the reproductive rate of the parasites (merozoites produced per asexual cycle) and (2) the number of parasites that lived and died as gauged from parasitemia counts such as that shown in Fig. 1. For comparative purposes, the stages of the 6 infections are drawn at the same scale although peak parasitemia varied from 4 days (see Figure 1) to a week or more because of differences in the number of parasites injected and the length of the asexual cycle. At the beginning of the infections, the merozoites produced per segmenter during each asexual cycle varied from 10 in *P. brasilianum* to 25 in *P. gallinaceum*, but only from 3 to 16 of these survived during the first asexual cycle. Particular attention is directed to the following: (1) In all 6 species of parasites there was a marked but temporary inhibition of reproduction and a marked increase in the number that died just after peak parasitemia. (2) *More* parasites died in the nonpathogenic species (A, B, D, E) than in the pathogenic species (C). The exception to this generalization (F) may be partly explained by the high reproductive rate of *P. gallinaceum*. (3) No discernible change in reproduction could be detected during minor relapses (A, B, D, F) except perhaps during the terminal stage of infections of *P. knowlesi* (C). Modified from Taliaferro (104) by permission of the author and the Williams and Wilkins Press.

peak parasitemia (arrows). This inhibition can be partly ascribed to athreptic innate factors, especially that occurring before peak parasitemia, in view of the work on malnutrition by Huff and his associates (43). On the other hand, parasite death was undoubtedly more pronounced after than before peak parasitemia in all six infections. The clearest-cut difference was encountered in infections of *P. brasilianum* (Figure 7A) and *P. lophurae* (Figure 7D). Whereas 64 to 70 per cent of the parasites died initially—that is, during innate immunity—96 to 98 per cent died just after peak parasitemia and about 90 per cent died (only about 1 out of 10 or 11 merozoites survived) during the developed infections, with occasionally a somewhat lower percentage dying (slightly more than one parasite surviving) during relapses. In three of the remaining infections (Figure 7B, E, and F), as compared to the death of 0 to 83 per cent during innate immunity, 94 to 98 per cent died just after peak parasitemia and slightly fewer (92 to 95 per cent) died during the developed infection. Infections of *P. knowlesi* (Figure 7C) in rhesus monkeys differed from those of *P. gallinaceum* (Figure 7F) in chickens only in the unexpected terminal survival of all the parasites produced.

Further inspection of Figure 7 indicates that the death of parasites was greater for such nonpathogenic species as *P. lophurae* than for the pathogenic species, *P. knowlesi.* The apparent exception to this generalization, the marked death of *P. gallinaceum,* which is lethal for many young chickens, is at least partially explained by the large number of merozoites it produced (25 at the beginning of the infection, as compared to 10 to 16 in other species).

Parasiticidal and reproduction-inhibiting activities of the host have also been analyzed with respect to acquired immunity in blood infections of certain trypanosomes. This analysis was carried out by obtaining parasite counts and indirectly measuring reproduction. The activities of the host with respect to innate immunity—that is, the suitability of the nonimmune host as a culture medium for the trypanosomes—were not determined because the total number of trypanosomes produced could not be ascertained for the asynchronously reproducing trypanosomes. The indirect measures for reproduction consisted in obtaining the percentage of dividing forms or coefficient of variation constants for size, since a high coefficient of variation of, for example, 20 per cent indicated growth stages and a low one of 3 per cent indicated no growth and no division. Such data during the course of various infections revealed the following: The mouse develops little or no *acquired* immunity against the so-called pathogenic trypanosomes *(Trypanosoma brucei, T. rhodesiense, T. equinum, T. equiperdum)*—it was found that the parasitemia increased logarithmically until the mouse died, while reproduction was maintained at a fairly constant high rate (94, 102, 119). Other hosts, like the guinea pig and the dog, develop lysins against the trypanosomes, but do not inhibit their reproduction, as is evidenced by recurrent increases and decreases in parasite populations while high rates of parasite reproduction prevail (72, 94, 108, 119). In contrast, the rat not only develops lysins but forms the reproduction-inhibiting antibody ablastin against *T. lewisi.* As a consequence, although *T. lewisi* rapidly divides and increases in numbers at first, it is nonpathogenic because it eventually cannot reproduce and is killed (93, 94, 96, 99, 102, 104, 108, 118, 119, 22). The mouse reacts somewhat similarly against *T. duttoni* (98, 102, 104, 116).

Parasiticidal factors have also been studied in various leishmaniases, especially by Stauber and his associates (1, 84), but the reproductive rates of the leishmania have not been measured.

Modifications of the host-parasite relationship offer an inviting field of study. Nonimmunological factors may greatly influence the course of infections. Some of the simplest procedures produced surprising results. As early as 1928, L. G. Taliaferro (92) delayed the highly synchronous cycle of *Plasmodium cathemerium* by placing parasitized blood in the icebox for 12 hours. During this interval the parasites apparently stopped growing, but when they were injected into canaries they proceeded to segment faster for a week until they were again segmenting on time. After Boyd (8) found that the timing of the cycle was controlled by light and dark, Stauber (83) used "dunce" caps to control the malarial cycle. He found that the cycle, especially the young trophozoite, was measurably affected by changing the temperature and periods of rest of the host. Hibernating squirrels are a unique host for study, as has been shown more recently by Jaroslow and his associates (11, 48). In addition, the course of some malarias is intensified by the parasites' preference for normal rather then sickled red cells (33) or for immature red cells rather than mature ones (81). This latter result was demonstrated by Singer (81) in an unexpected manner in X-ray experiments on the fatal infections of *Plasmodium berghei* in mice. In mice whose hematopoietic system was injured by 550 R, the parasitemia reached a peak in 5 days and then declined to a subpatent level because of a lack of immature red cells, whereas in control unirradiated mice it mounted for 2 weeks or more. Goble and Singer (37) studied the effect of daily intravenous injections of such substances as Thorotrast, saccharated iron, or polyvinyl pyrrolidone in mice at the beginning of infections of

Plasmodium berghei or of *Trypanosoma congolense*. They found, on the one hand, that Thorotrast enhanced the malarial and trypanosome infections—that is, depressed innate immunity—whereas some of the other materials prolonged the trypanosome infections but only suppressed the initial minor malarial crises while not delaying the final fatal outcome. These authors (37) thoroughly reviewed previous work designed to modify the course of various malarias and trypanosomiases, and Goble (36) reviewed the immunoreactions in antiparasitic chemotherapy.

Irradiation at critical times has also been found to suppress immunity against certain infections (133). For example, Jaroslow (46, 47) infected mice with nonpathogenic *Trypanosoma duttoni* 14 days before to 22 days after 550 R. He found that all the mice died with overwhelming parasitemias and high reproductive rates when infected from 4 days before to 15 days after 550 R, but showed little change in their infections when infected 14 days before or 22 days after 550 R. An analysis of the data indicated that X-rays markedly suppressed the formation of anti-*duttoni* antibodies, that the reproduction-inhibiting capacity of the host was more sensitive to X-ray injury than the trypanocidal activity, that both activities were resistant if antibody titers were high (infection 2 weeks before X-rays) and that recovery from X-rays began in three weeks (infection 22 days after X-rays). These findings fall in line with irradiation studies on the hemolysin response as reported by my associates and me (124, 125, 131, 132). Somewhat similar results were reported previously for *T. lewisi* by Naiman (66) and for *Plasmodium gallinaceum* and *P. lophurae* by us (135). From our results, we concluded that an X-ray-induced decrease in immunity, as gauged by increases in parasitemia, is only detectable when the sum total of innate and acquired immunity is at an intermediate level. Thus, a dose of 550 R to mice a week after infection with *T. duttoni* caused a relapse (intermediate level of immunity), but not when given 2 weeks after infection (strong immunity).

Delayed hypersensitivity

The problem of delayed hypersensitivity is surrounded by perplexities and the absence of quantitative measurements. The phenomenon develops slowly as a lesion over a period of 24 to 72 hours at the site of antigen deposition in a sensitized animal and in the absence of circulating antibody. Moreover, it can only be passively transferred, by cells—not by serum—from peritoneal exudates of lymphoid tissues of a sensitized animal (31, 34, 44, 54, 55). As far as I am aware, it has not been induced by protozoa, but has been developed in the guinea pig to *Trichinella spiralis* larval antigens (54, 55).

The parasite

The humoral activities of the host depend upon what parts of the parasites act as effective antigens. Early in this century, the antigenic character of African pathogenic trypanosomes was studied with respect to relapse variants (95). Ritz (77) found 22 immunological variants of *T. brucei* in 600 mice, and one of his mice, which was incompletely cured with drugs 20 times, produced 17 immunologically different relapse strains. The differences encountered were based on the fact that a mouse cured of an infection with a given pathogenic trypanosome by drugs is refractory for about 20 days to a second infection of the same strain. In 1963 Weitz (33) summarized work on the antigenicity of some African trypanosomes, and in the same year Brown (33) summarized work he and his collaborators have been engaged in on the characterization of the *Trypanosoma brucei* antigens by various chemical, physical, and immunological methods. Zuckerman (33, 140, 141) has likewise been undertaking a systematic study of antigens in malaria.

Canning in 1929 (13) was the first worker to study the antigenic mosaic of helminths. Before there was any general interest in analyzing the mechanisms of immunity to these parasites, he found antigenic similarities and differences in such isolated tissues as egg, sperm, muscle, intestine, and cuticle of ascaris.

He concluded that certain of these were better suited for use in immunological tests than the whole worm, where conflicting elements would obscure the results. More concerted attacks have been carried out within the last ten years. For example, as has been reported by Kagan and his coworkers (52, 53), with sheep hydatid fluid globulins of *Echinococcus granulosus* separated by immunoelectric methods and tested by gel diffusion techniques, 10 of 19 detectable antigens were of sheep-serum origin and could be removed by absorption; in similar tests with human hydatid fluid, 4 of 23 detectable antigens were of parasitic origin, 6 of host origin, and 13 of undetermined origin. In a comparable study, *Toxoplasma gondii* showed 14 host components and 3 to 4 parasite components (19).

Work on the biochemistry of plasmodia, including metabolic pathways and nutritional requirements, has been reviewed from a stimulating point of view by Moulder (64), and the chemical composition and metabolism of protozoa—chiefly the free-living protozoa—have been brought into focus in the review by Seaman and Reifel (79). The recent monograph on the biochemistry of parasites by von Brand (9) describes the newer trends in dealing with biochemical aspects of parasitology, including intermediate carbohydrate and protein metabolism. Further study on the mechanisms underlying antigenic variations needs the coordination of serological, biochemical, and genetic approaches, as Beal and Wilkinson note (5). Such studies, in addition to their intrinsic value, may bring to light common antigens in the parasite and host that may interfere with host resistance. Host mimicry has been discussed by Damien (23).

The hemolysin response

The difficulties inherent in demonstrating humoral phases of host-parasite relationships can be circumvented in other antigen-antibody reactions. It was for this reason that we started work on the hemolysin response induced by a nonreproducing, foreign, benign antigen, which can be accurately titrated by colorimetric methods (12, 121, 127). I should like to describe briefly a few salient results that we have obtained with this response since we are now in agreement that the host reacts in a somewhat stereotyped manner against all foreign proteins.

The hemolysin response can be induced in rabbits by the intravenous injection of sheep red blood cells (sRBC) containing the Forssman antigen. As is shown in Figure 8, the response is characterized by a latent period when no hemolysin can be detected in the serum, a rapid rise of hemolysin to peak titer, and a subsequent less rapid decline. This curve is remarkable in that the individual segments are more or less linear and allow various parts of parameters to be measured for times, rates, and peak titer. Peak titer is important because it gives a relative measure of the amount of hemolysin formed.

The most spectacular result we found was that the latent period can be divided into two

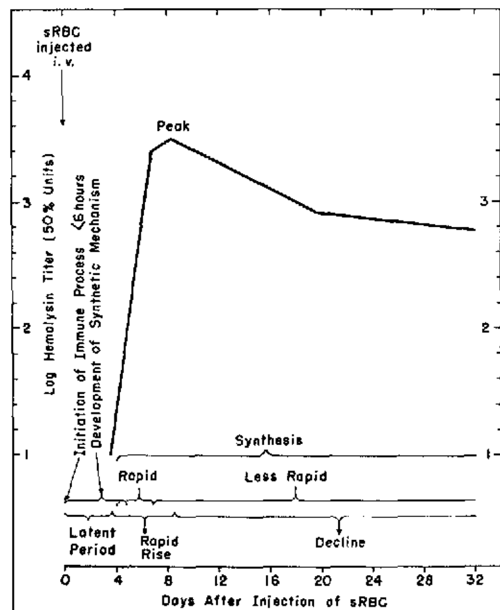


FIGURE 8. The mean hemolysin response in a group of rabbits following one intravenous injection of 10^9 sheep red blood cells (sRBC) per kg rabbit, as ascertained by hemolysin log titer (determined colorimetrically in 50 per cent units). After a latent period, hemolysin rose rapidly to peak titer and then declined. From data of Taliaferro and Taliaferro.

parts. Moreover, the first part—induction of antibody cells by antigen—occurs in an extremely short time and determines the amount of hemolysin formed; the second and much longer part involves the elaboration of the antibody-synthesizing mechanism, which thereafter works rapidly at first and more slowly later. These results were obtained by using radiation as a dissecting tool. Parenthetically, it should be emphasized that the following results in rabbits are based not only on determining the timing and dosage of X-rays (124, 131, 132), assembling and testing adequate serum samples over a sufficient length of time to show lengthening latent periods, and so forth, but on a thorough knowledge of the response in unirradiated controls with respect to their variability when similarly treated (121-123, 129, 130), the suitability of a given amount of antigen (122, 130), and the route used to introduce it (28). Such variables have to be reassessed when mice, rats, or other species are studied (133).

Figure 9 illustrates pertinent data obtained from the irradiation experiments. The mean control response when only red cells were given (Figure 8) is repeated in each section of this figure for comparative purposes. Thus, after a latent period of 4 days, hemolysin rose rapidly to a peak titer of 3.5 log units on day 8.4. In marked contrast, hemolysin in rabbits given sheep red cells 4 hours after 500 R did not appear in the serum for 8.9 days and only reached a titer of 2.5 log units on day 19.3

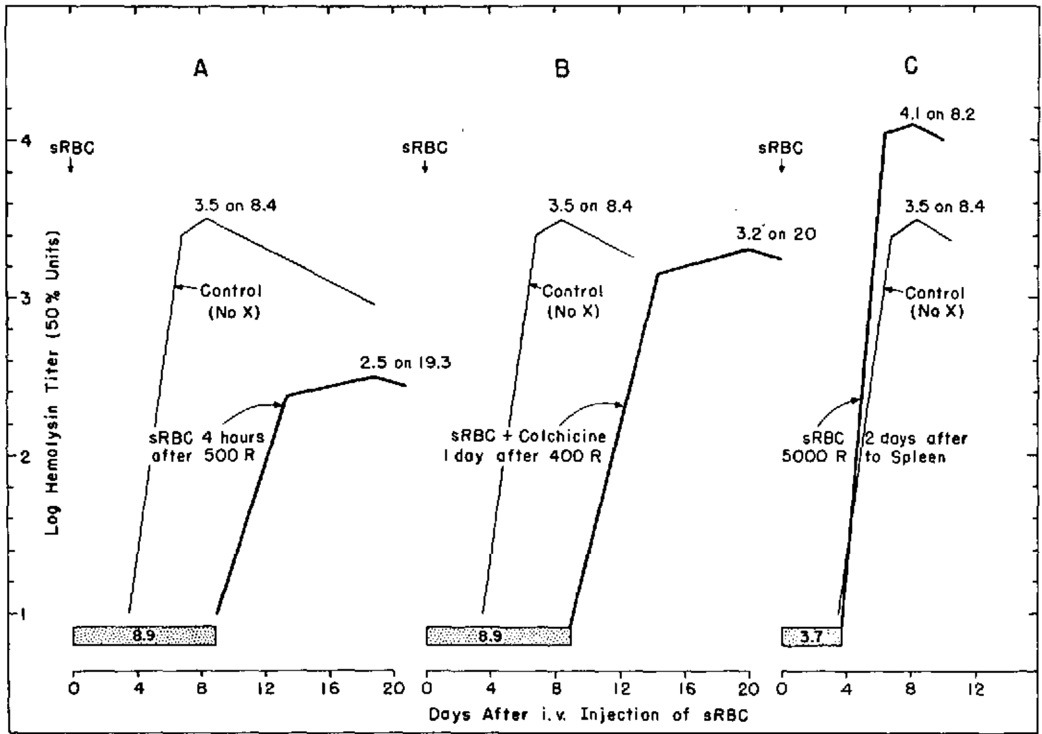


FIGURE 9. The mean hemolysin response in 3 groups of rabbits following one intravenous injection of 10^9 sheep red cells (sRBC) and variously irradiated as compared to the mean hemolysin response in unirradiated rabbits (Control: no X). From data of Taliaferro and Taliaferro.

A. In irradiated (500 R) rabbits given sRBC 4 hours later, the latent period was lengthened and the peak delayed and decreased.

B. In irradiated (400 R) rabbits given sRBC + toxic doses of colchicine 1 day later, the latent period was lengthened but the peak titer was almost completely restored.

C. In spleen-irradiated (5000 R) rabbits given sRBC 2 days later, the latent period was normal in length and peak titer was enhanced.

(Figure 9A). Here, induction and the synthetic mechanism were both injured (125, 131, 132). When, however, sheep red cells plus large toxic doses of colchicine were given to rabbits 1 day after 400 R, hemolysin rose to a peak (3.2 log units) practically as high as the control value, but only after a long latent period of 8.9 days (Figure 9B). Here induction was restored but the antibody-synthesizing mechanism remained injured (49, 50). Finally, when sheep red cells were injected into rabbits 2 days after the spleen alone was irradiated with 5,000 R, hemolysin rose to a remarkably high level after a latent period comparable to that in the control. Here not only were both parts of the latent period restored, but stimulation occurred (88, 127).

Jaroslow and I (49, 50) concluded from these and other irradiation experiments that —either directly or indirectly—the materials or procedures that restore induction release nucleic acid degradation products that are in short supply in the host. These materials in some way facilitate induction of certain primitive mesenchymal cells of various lymphatic tissues (16, 91, 129, 139).

The antigenicity of sheep red cells was also studied. Talmage and I (136) found that two Forssman hemolysins arise as a result of injecting heated sRBC and that these two together with two isophile hemolysins arise as a result of injecting fresh sRBC. These antibodies differ in several respects. When anti-Forssman hemolytic serums were separated electrophoretically, 1 cm fractions in starch blocks 50 cm long showed two peaks of hemolysin in the globulin area. The fast-moving globulin with a peak at 38 cm, which was identified as the IgM (γ_1) component, always predominated; the slow-moving one with a peak at 44 cm, which was identified as the IgG (γ_2) component, was proportionately small in amount (0.2 per cent) during initial immunization but increased to 10 per cent or more during hyperimmunization. Moreover, the large IgM hemolysin, with a molecular weight of about 900,000, appeared early in immunization, was markedly avid, and decayed with a half life of 2.8 days, whereas the small one, with a molecular weight of 160,000, appeared late in immunization, was only moderately avid, and decayed with a half life of 5.6 days. These data are shown in Table 1. Thus, in the rabbit, the predominant IgM Forssman hemolysin appears in detectable amounts sooner than the IgG Forssman hemolysin. Moreover, it seems probable that both the IgM and IgG isophile hemolysins appear late in immunization. The structure and biological activities of other immunoglobulins have recently been inten-

TABLE 1. Characteristics of IgM (γ_1) and IgG (γ_2) Forssman hemolysins in rabbits injected with heated stromata from sheep red-blood cells

	Separation		Reference *
Characteristic	IgM (γ_1)	IgG (γ_2)	a, e, g, h, j
Appearance and peak titer (after immunization)	Early	Late and especially in hyperimmune animals	c, d, g, h, i, j
Molecular weight	900,000	160,000	a, d, g
Rate of hemolysis	Varies as the square of of the concentration	Varies as the fourth power of the concentration	f
Blood/tissue equilibration	80/20	50/50	b
Half life	2.81 ± 0.12 days	5.56 ± 0.17 days	b
Avidity	High	Moderately low	f, i, j
Action of 2-mercaptopurine	Degraded	Not degraded	g

* Data from (a) Stelos (1956); (b) Taliaferro and Talmage (1956); (c) Talmage *et al.* (1956a); (d) Talmage *et al.* (1956b); (e) Stelos and Talmage (1957); (f) Weinrach *et al.* (1958) and Weinrach and Talmage (1958); (g) Stelos and Taliaferro (1959); (h) Stelos *et al.* (1961); (i) Taliaferro, Taliaferro and Pizzi (1959); (j) Taliaferro and Taliaferro (1961). These papers may be found in one or more of the following references: 86, 87, 110, 128, 133, 134, 136.

sively studied (18, 32, 35, 69).

The foregoing characteristics of the hemolysins against sheep red blood cells should be considered in searching for antibodies against parasites, especially when parasites inhabit red blood cells or contain antigens with a Forssman specificity. Other facets of the hemolysin response may with profit be considered a pattern upon which to establish likenesses and differences in the various host-parasite relationships, and the effects of irradiation and the restorative procedures may be a touchstone for assessing future parasitological work.

Summary

These introductory remarks are concerned with past accomplishments. Parasitologists during the first quarter of this century were mainly interested in systematics and life histories, and some of them, especially helminthologists, were disinclined to recognize that acquired immunity develops against the animal parasites. Soon thereafter, however, the immunology of parasitic infections began to be brought into focus; it gathered momentum during the second quarter of the century chiefly because changes in the blood populations of plasmodia and trypanosomes could be related to host reactions. The cellular activities of the host were explored, and the developmental potencies of the lymphocyte began to be realized—although in this field as in others, many were disinclined to attribute any function at all to this cell. During the middle of the century immunological parasitology flourished. Of the subjects investigated, differences between pathogenic and nonpathogenic infections were clarified by separating parasiticidal from reproduction-inhibiting mechanisms and by modifying infections in various ways.

Basic data on the hemolysin response in rabbits after the intravenous injection of sheep red blood cells are described in order to give a general idea of the rise and fall in antibody formation in a system with two distinct advantages. This system is initiated by a nonreproducing benign antigen, and serum antibody can be accurately measured photocolorimetrically. The results from these data are paralleled by certain results obtained in parasitic infections.

The third quarter of this century promises steady advances on problems related to the antigenic mosaic and biochemistry of the parasites, and critical analyses of the cellular and humoral activities of the invaded host. These advances will undoubtedly rest on the use of new methods involving the electron microscope, genetic and isotopic markers, autoradiography, and electron microscopy. As biochemical phases of parasitism are developed, we should be better equipped to understand the basis of nonantibody and antibody immunity.

Moderator: Dr. Taliaferro's presentation has given us a remarkable example of the need for basic knowledge and applied knowledge to go hand in hand. It was also very inspiring to hear how, starting from the study of applied immunology, Dr. Taliaferro could make such a contribution to basic immunology.

Let us now have the papers by Dr. K. Neil Brown and Dr. Irving G. Kagan, and then discuss both these topics afterward.

NATURE AND VARIATION OF PARASITE ANTIGENS

K. N. Brown

Introduction

The life history of protozoa and helminths parasitic in vertebrates usually includes a vector and, with some helminths, free-living stages as well. These separate habitats, together with the need for transmission and sometimes the localization of the parasite in a number of distinct tissues, are all factors that require some particular specialization in parasite structure and physiology.

In protozoa, this adaptation occurs at the unicellular level, but in helminths multicellular organization is involved. Cellular differentiation produces tissue-specific antigens in mammals (21), and, equally, each specialized form of the parasite has certain unique antigenic characters. In addition, at least three species of protozoa can change their antigenicity repeatedly without visible alteration in cell structure.

This paper will discuss the capacity of parasite antigens to vary in one life cycle. In order to underline the relevance of parasite specialization and adaptability to the immunology of parasitic infections, emphasis will be placed on antigenic differences rather than on similarities. Vector- and free-living forms will be compared with the vertebrate-infecting organism, and then the various types of parasite developing within the vertebrate will be considered.

Vector- or free-living and vertebrate-dwelling forms

Brucei-group trypanosomes occur as "tsetse mid-gut trypanosomes," "crithidia," and "metacyclics" in the vector and in another "trypanosome" form in the vertebrate. Besides being different in shape, the "mid-gut" and "blood" trypanosomes are known to show differences in their respiratory systems (27) and lipid content (Dixon, personal communication). Antigenically they are very different. Immunization with the mid-gut parasites gives no protection against the blood forms (Pittam, personal communication), and only two of the many precipitinogens present in the blood trypanosomes occur in the mid-gut parasite (39, 28). In fact, blood trypanosomes from two separate strains may have more in common than mid-gut and blood forms of the same strain (28).

In *Leishmania donovani*, the insect-dwelling leptomonads and the vertebrate-dwelling leishmanoids show respiratory differences analogous to the *Trypanosoma brucei* mid-gut and blood trypanosomes (16). Leptomonads, however, convert directly to leishmanoids, and this transmission can be accomplished *in vitro* by increasing the temperature from 25° to 34°C (17). A similar leptomonad-to-leishmanoid conversion follows *in vitro* treatment with anti-leptomonad serum (2), and the two stages give distinct agglutination reactions (8, 3). *T. cruzi* crithidia, normally found in the triatomid vector, can also be modified *in vitro* by immune serum to produce the vertebrate-infecting trypanosome and leishmanoid parasites, leishmanoid production requiring a higher concentration of antiserum than the leptomonad-to-trypanosome change; a single flagellate in immune serum can produce colonies of trypanosomes and leishmanoids (1). Like the change from leptomonad to

leishmanoid in *L. donovani,* the crithidia-to-trypanosome conversion can be reproduced experimentally *in vitro* by raising the temperature—from 26° to 37°C (35). In the normal life cycle these changes are probably physiologically induced, but elimination of the *T. cruzi* trypanosome parasite to leave only leishmanoids occurs when acute Chagas' disease gives way to the chronic condition, presumably as the result of host immune reactions.

In malaria, immunization with sporozoites of *Plasmodium gallinaceum* isolated from mosquitoes gives some protection against sporozoite challenge but none against erythrocytic parasites (24). Fowls immunized with erythrocytic forms are somewhat protected against erythrocytic and sporozoite challenge, not an unexpected result since the infection progresses from the sporozoite to the erythrocytic stage.

Antigenic differences between vector- or free-living and adult helminths have also been described. The reaginic antigen of *Nippostrongylus braziliensis,* a "metabolic product," is present in adults and fourth-stage larvae but only in trace amounts in the free-living third stage (22). In *Schistosoma mansoni,* protective immunity develops in rhesus monkeys after intravenous transplantation of adult worms, but monkeys immunized with schistosome eggs are not protected (33). Egg and cercarial antigens can be differentiated from adult antigens by gel diffusion (31).

Antigenic changes within the vertebrate

Once the parasite is inside the vertebrate, morphological and physiological specialization continues and further antigenic changes follow. These developments can be divided into two types: (1) those inherent in the life cycle, like the sporozoite, pre-erythrocytic, and erythrocytic progression of malaria; and (2) those that can apparently occur as a result of the host's immune response, for example the *T. cruzi* trypanosome to leishmanoid transition referred to above.

1. There is only limited evidence on changes inherent in the life cycle. Pre-erythrocytic and erythrocytic *P. vivax* may be antigenically distinct, since pre-erythrocytic parasites can develop even if subsequent blood invasion is prevented in the immune host (29). X-irradiated *Schistosoma* cercariae can develop into stunted schistosomula but still fail to immunize effectively as transplanted adult worms (32, 33).

2. *In vivo* modifications of parasite structure, apparently associated with host immune reactions, have been reported in several species. The trypanosome-to-leishmanoid transition of *T. cruzi* has already been cited, and host immunity also alters the structure of *Toxoplasma* (37). In both these species the modified parasites tend to remain together in "nests" or "pseudocysts," and *T. cruzi* cultivated *in vitro* in immune serum forms clumps and syncitia (1). Since the modified parasites survive, they are, by inference, antigenically different from the form they replace, although their distribution within the host's tissues may affect their susceptibility to immune reactions.

The immunological environment may also modify helminths, for worms in immune animals may be alive but stunted. Host immunity presumably inhibits parasite physiology and development, and antigenic substances that are normally produced by the adult worm but would be lethal to the parasite in an immune host may fail to develop fully; possibly analogous stunting and cellular modification occurs in embryonic bone cultivated in immune serum (10).

Therefore, the structure, the function, and probably the antigenicity of parasites can be modified by immunity. Additional antigenic changes not involving visible morphological alteration occur in at least three protozoan species. This phenomenon, antigenic or relapse variation in the classical *brucei* trypanosome sense, will be described in the next section.

Classic "brucei-type" antigenic variation

"*Brucei*-type" variation is used here to mean repeated antigenic changes similar to those described for *T. brucei* over fifty years ago (26). Similar variation was later shown in *T. congolense* (12) and more recently in simian malaria (6). The variants are morphologically indis-

tinguishable, but *brucei* variants are known to show some differences in drug sensitivity (14) and in the electrophoretic mobility of their major variant-specific antigens, the 4S group of proteins (39, 18). Variation occurs continually in chronic infections, and over twenty variants have been described for one strain of *T. brucei* (25); in some experimental hosts protection may be completely variant-specific (12). After cyclical development in the tsetse, variants tend to revert to a "parent" serotype that appears as the first parasitemia following an infective bite (4, 13). Because of the continuous variation, random isolations are of limited value when strains are being compared, but the immediate post-tsetse parasites have been used with some success (13).

Variant trypanosome antigens can be detected by agglutination, lysis, protection, and precipitin tests. In *P. knowlesi* malaria, on the other hand, only an agglutination reaction with schizont-infected cells has been used. Erythrocytes infected with immature trophozoites do not agglutinate, which suggests that the schizont develops antigen that the trophozoite lacks, or, alternatively, that the breakdown of red-cell structure that accompanies parasite maturation allows parasite antigenic material to reach the red-cell membrane surface (7). Schizonts freed from erythocytes by immune lysis with goat anti-monkey red-cell serum barely agglutinate in anti-malarial serum, so perhaps the agglutinogen consists of parasite and red cell components; a combined parasite and red-cell antigen has been described in *Anaplasma* (30). Gametocytes of *P. knowlesi* also may show antigenic variation (15), and the effect of vector transmission on antigenicity could provide an interesting comparison with the asexual *brucei* trypanosomes.

By repeated antigenic changes of the *brucei* type, parasites can persist for many months in one host. Chronic infections are characteristic of many protozoan diseases, which indicates that *brucei* variation may be common in protozoa. Variation may not be confined to one form of the parasite—in *T. cruzi*, for example, it may perhaps occur in both trypanosomes and leishmanoids. Nevertheless, in spite of variation, parasites usually become increasingly scarce as immunity develops, although they remain fully virulent for the nonimmune animal. Some of the factors involved in this generalized partial immunity have been discussed elsewhere (5), but it may depend in part upon antigenic determinants shared by all variants. In antigenic studies on protozoa, a clear distinction between "common" and possible "variable" antigens is necessary. "Common" antigens may or may not be shared by different morphological forms of the parasite.

With the more complex multicellular helminths, antigenic modification without morphological change is more difficult to visualize, but this possibility cannot be excluded. Recent experiments show that adult schistosomes transferred from hamsters to rhesus monkeys die or fail to produce eggs although rhesus-to-rhesus transfers are fully fertile (33). These results could be interpreted as showing that the primitive cells differentiating in the developing schistosomula are influenced or selected by host antibody in such a way that only molecular configurations nonantigenic in that particular host develop. An immune response would occur on transfer to another species of host. Possible similarities have been suggested between host and parasite antigens in another context (34, 9).

General considerations

Although the antigenic structure of most protozoa and helminths remains unknown, their life cycle may include several, and sometimes many, antigenic forms. Antigenic change may follow obvious structural reorganization stimulated by the physiological and immunological environment, but in at least three species—*T. brucei*, *T. congolense*, and *P. knowlesi*—repeated antigenic variation occurs without obvious alteration in morphology. The extent and significance of this antigenic liability must be recognized and fully characterized before we can understand parasite immunology. Several interconnecting lines of approach are possible

and desirable; they include examination of the following:

1. In any given species, the degree of variability occurring in a life cycle, particularly within the vertebrate.

2. The extent to which total antigenic structure is involved in any changes that occur.

3. The comparative protective value of these antigens.

4. The effect of vector transmission on the antigenic repertoire of the parasite.

5. The comparison of strains in relation to their geographical distribution, using a fixed point of reference in the life cycle.

6. Antigen immunochemistry and its relationship to parasite physiology, including the comparative value of live and dead antigen, and the possible role of "exo-antigens." The use of live parasites of known antigenic character inhibited by mitomycin or actinomycin (11) might prove very revealing.

7. The character of the immune response stimulated by each stage of the parasite. The response will be related not only to antigenic structure but also to the location of the parasite within the body. For example, cutaneous leishmaniasis produces a delayed skin reaction but no demonstrable antibody, while visceral leishmaniasis produces antibody but no delayed response. These effects are presumably due to the contrasting localized and systemic type of infection. An understanding of this aspect is essential for a full appreciation of the antigenic potential of parasites.

The experimental approach to these problems involves four fundamental points:

1. Parasites must be maintained under constant conditions, with strain histories recorded in full. Where possible, reference "stabilates" (20) must be established to which all parasite isolations may be related. In protozoa this can be done by keeping viable parasites in deep-freeze (23). Where strains are maintained *in vivo,* the animal species used for keeping the strain should, for preference, be different from the experimental host. Parasite antigens may come to mimic host antigens (34, 9), and they may not be detected if strain and experimental hosts are of the same species.

2. The development of suitable immunological tests. Classic serological techniques may not be sufficient, and host cells, for example macrophages in toxoplasmosis (36), may be required. Although *in vitro* tests may not always correspond directly to events *in vivo,* they have one advantage in the initial stage of an investigation: that they concentrate observation on a particular selection of parasites, antibodies, and host cells collected at a defined time. *In vivo* experiments, preferably in inbred animals, are likely to prove most valuable where cell-mediated immunity is suspected; examples include coccidiosis (19) and cutaneous leishmaniasis. Host species vary in their response to parasitic infections; therefore the experimental animal chosen should, if possible, suffer a similar type of infection to that occurring in man or the pertinent domestic animal. In some instances it may even be preferable or necessary to use an alternative species of parasite as a model.

3. The isolation of parasite material free of host cells. This is often difficult, but a new and improved technique has recently been devised (40). The possibility of host cell components' being included in parasite antigenicity must be considered.

4. Micromethods of analysis. Techniques involving live parasites or their constituents will often depend on small amounts of material. Microscopic observation of parasites in experimental situations will be necessary, and in immunochemical analyses some newer micromethods (38) should prove valuable.

In any experimental approach, many more factors will have to be considered than those outlined here, and each parasite will set its own technical limitations. Nevertheless, the points mentioned, some of which are necessarily fairly obvious, are likely to contribute to a full analysis of protozoa and helminth parasite antigens.

CHARACTERIZATION OF PARASITE ANTIGENS

Irving G. Kagan

The biological and immunological activities of parasite antigens have been under investigation since the turn of the century, and antigen-antibody interactions in helminthiases, particularly, have been discovered to be many and complex (111). Hydatid fluid from cysts of *Echinococcus granulosus* was used as an antigen in the complement-fixation (CF) test in 1906 (39). Since then parasite serology has grown in variety of tests standardized and in kinds and types of antigens employed. Many serologic tests have lacked specificity, but today we do have specific tests for a number of parasite infections (50). There is still the need for improvement. Almost without exception, the serologic antigens employed have been mixtures of many components. Research to isolate and characterize diagnostic parasite antigens has been made. Some of these studies will be reviewed.

The use of parasitic antigens has not been limited to serology. They have also been used as vaccines to stimulate host resistance. Initially, crude homogenates of parasite material were injected to stimulate immunity. Early investigators differentiated between the somatic antigens obtained from the body of the parasite and the secretory and excretory antigens of the living organism. The latter were believed to be the important ones in immunity. With improved techniques for antigenic analysis, the differences between these two types of antigens became less significant. Today we group parasitic immunogens into "functional" and "nonfunctional" antigens. Soulsby (112) very skillfully reviewed this subject. The functional antigens are the ones that interest us, and when we have isolated and characterized them fully we may be able to synthesize them or attach a synthetic immunogenic group to a biological carrier for vaccination purposes.

Dineen's (30, 31) and Damian's (26) provocative speculations on the host-parasite relationship suggest that the immune response of the host may exert a selective pressure on the parasites that have less antigenic disparity with the host. The parasite can then be thought of as a successful tissue graft that does not stimulate a rejection response on the part of the host. In a successful host-parasite relationship many antigenic determinants must be shared between the parasite and the host. If this is true, then "eclipsed" antigens and "molecular mimicry" between parasite and host have broad biological significance. Differentiating between host and parasite components becomes important in developing specific antigens for serologic and immunologic studies.

Nonspecific, cross-reacting parasitic antigens

Antigens with broad specificity in helminthology are the polysaccharides of numerous species that exhibit blood-group activity. The biological activity of these antigens was reviewed by Oliver-González (87), who has contributed many of the observations in this area. A more recent review was made by Damian (26). Campbell (19) analyzed the polysaccharide of *Ascaris suum* and found hexoses and glucose but no hexuronic acid, pentoses, ketoses, or manoses. Kagan *et al.* (57) were also unable

to find pentose in polysaccharide extracts of *A. suum*. Ascaris polysaccharide is reported to have blood group antigens of the ABO system (86, 113).

Oliver-González and Kent (89) present evidence that the A_2 isoagglutinogen-like substance prepared from the cuticle of *A. suum* is serologically related to *Clostridium* collagenase. They assayed the Ascaris material by specific action and degree of inhibitory activity against A_2 isoagglutinins in human sera of blood groups O and B, in hemagglutination tests with antisera against the blood-group factor and cells coated with collagenase, and by gel diffusion analysis. This is one example of cross-reacting antigenic substances found in phylogenetically distantly related organisms that react antigenically in serologic tests. The collagenase from the *Clostridium* and the collagenase-like extract from the cuticle of *A. lumbricoides* killed dogs with an anaphylactoid reaction, and both caused similar histopathology, as was seen at autopsy.

Insight into the antigenic nature of some parasitic materials has been derived by inference and not by direct isolation and characterization. Another example of an antigen shared between a helminth and a microorganism is the relationship between *Trichinella spiralis* and *Salmonella typhi* (124, 125). Since the antigenic configuration of *Salmonella* species is known, various *Salmonella* were reacted with an anti-trichinella serum in an agglutination test. The major cross-reacting antigens involved in these tests were the somatic 12 antigen of *Salmonella* and, to a lesser extent, the somatic 9 antigen. The somatic 12 *Salmonella* antigen successfully immunized mice and rats against experimental infection with larvae of *T. spiralis*. The somatic 12 antigen of *S. typhi* has been characterized as having two molecules of carbohydrate, one terminating in glucose and the second in rhamnose (71).

Another instance of cross-reactivity between *Ascaris* and pneumococcus *(Diplococcus pneumoniae)* was described by Heidelberg *et al.* (45). Glycogen of *Ascaris* is thought to be closely related to mammalian glycogen composed of 12-13 glucosyl chains linked α (1-6) with many α (1-4) branch parts and with an average molecular weight on the order of 9 x 10^6. Due to the 1-4, 1-6 linkage, *Ascaris* glycogen will cross-react with various pneumococcal antisera.

An antigenically active polyglucose was isolated by Sawada *et al.* (98, 99) from *Clonorchis sinensis*. The antigen was isolated after delipidization with diethyl ether and extraction in distilled water. The concentrated material was then passed through a Sephadex G-100 column, a CM-cellulose column, and a DEAE-Sephadex A-50 column and deproteinized by 90 per cent phenol extraction. The purified carbohydrate antigen contained 90.6 per cent glucose and perhaps 1 per cent pentose, plus negligible amounts of nucleic acid and phosphorus. On infrared spectographic analysis the polyglucose of *C. sinensis* gave a pattern almost identical with a polygluclose isolated from *Mycobacterium tuberculosis*.

Antigens from mycobacteria cross-react in *Leishmania* serologic tests (83). A recent report (129) indicated that BCG could be substituted for the *Mycobacterium butyricum* previously used in serologic tests for leishmaniasis.

Since Yamaguchi (130) reported the Forssman antigen in the larvae of *Gnathosoma spinigerum* in 1912, other parasitic worms have been shown to contain it, including the larvae of *T. spiralis* (78), the third-stage larvae of *Oesophagostomum dentatum* (110), *Hymenolepis diminuta* (43), and *Schistosoma mansoni* (88, 28).

The presence of C reactive protein in at least 13 species of helminths, including nematode, trematode, and cestode species, was demonstrated by Biguet *et al.* (12). C reactive protein is distributed quite widely in the animal kingdom.

The occurrence of cross-reacting antigens in parasites of different species may be due to a number of causes. Most obvious is the cross-reactivity to be expected if the parasites are phylogenetically related. Another reason may simply be the chance occurrence of similar

antigens among unrelated organisms. However, if the parasites have hosts in common and therefore are ecologically related, cross-reactivity may have yet other bases. Two alternative hypotheses for this phenomenon were recently advocated: Damian (26) suggested that convergent evolution of eclipsed antigens may be responsible, and Schad (100) proposed that the development of nonreciprocal cross-immunity may have a significant effect on the distribution of a parasite. By possessing cross-reacting antigens, one parasite may exert a limiting effect on another's distribution through the agency of the host's immune response. Several examples of such parasitic relationships are reviewed in his paper.

Host antigens present in the parasite may constitute a final area of nonspecificity. Kagan *et al.* (58) demonstrated that serum of patients ill with a number of collagen diseases contained antibodies that cross-reacted nonspecifically with host antigens found in echinococcus hydatid fluid.

Chemical identification of helminth antigens

The chemical identification of parasite antigens has followed an empirical course. In most instances, techniques that have proved useful in the isolation of microbial antigens have been employed.

The antigenic components active in the CF test for schistosomiasis have been investigated by several groups. Rieber *et al.* (93) separated adult worms into lipid, carbohydrate, and protein fractions. As expected, two of the five lipid fractions fixed complement with syphilitic serum but were inactive with schistosome antibody. The carbohydrate fraction was nonreactive. The acid-insoluble protein fraction (which can be precipitated in 30 per cent saturated ammonium sulfate) contained the antigenic component. This antigen was electrophoretically homogeneous. Sleeman (104) extracted schistosome adult worms with sodium desoxycholate, a reagent also used by Schneider *et al.* (102), followed by fractionation with ethanol and precipitation with calcium. On chemical analysis this antigen was found to contain protein and lipid in a ratio of 2.5:1. The purified antigen was free of carbohydrate and after acid hydrolysis was negative for purines and pyrimidines. Since Cohn's method for isolating fraction III-O was employed, Sleeman suggested the antigen may be a beta-lipoprotein or a "lipo-poor euglobulin."

An antigenic polysaccharide material was extracted from cercariae and eggs of *S. mansoni* by Smithers and Williamson (107, 127). Extensive analysis indicated that the antigen was a "glucan polysaccharide of glycogen-like properties." A similar antigen was prepared for the intradermal test by Pellegrino *et al.* (92) from cercariae of *S. mansoni.* These workers concluded from their studies that chemical components other than carbohydrates were active in the schistosome skin test. Kagan and Goodchild (55) evaluated the polysaccharide content of a series of antigens that were adjusted to similar nitrogen content and gave similar reactivity in the skin (the wheal areas in 25 infected individuals were not significantly different). The carbohydrate content did not correlate with the intradermal activity. Gazzinelli *et al.* (38) fractionated cercarial extract in a DEAE-Sephadex A-50 column and found the most active fraction in the intradermal tests to be free of polysaccharide.

A lipoprotein was isolated from *Fasciola hepatica* by precipitation with dextran sulfate; final purification was by differential ultracentrifugation in a high-density salt medium. Immunochemical electrophoretic analysis indicated a pure fraction. The antigen was immunogenic and similar in chemical composition to alpha lipoprotein of human serum. The active lipoprotein constituted 2 per cent of the worms' dry weight; it had a sedimentation constant of 4.9S and a molecular weight of 193,000 (65-67).

Maekawa and Kushibe (73, 74) isolated and characterized an antigen from a heated extract of *F. hepatica* by means of precipitation by ammonium sulfate and phenol treatment followed by extraction with potassium acetate

and ethanol. One of the antigenic components was further analyzed and found to be composed of ribonucleic acid (95 per cent) and small amounts of peptides. This antigen was a potent intradermal reagent in cattle (75) and was earlier crystallized by these authors (76). A serologic antigen devoid of protein- and lipid-containing polysaccharide material was prepared by Babadzhanov and Tukhmanyants (5).

Protein complexes of helminths have been under active study. Kent (59) reviewed his early work on the isolation of proteins from *Moniezia expansa, Hymenolepis diminuta,* and *Raillietina cesticillus*. In his studies on *A. suum* (60, 61) five protein fractions were isolated by DEAE cellulose chromatography. The fractions were all glycoprotein complexes containing glucose and ribose with different amino acids. The two fractions with the highest carbohydrate content were the most antigenic. Working with larvae of *T. spiralis*, Kent (62) isolated four antigenic glycoprotein fractions by column chromatography.

The antigens of *T. spiralis* have been studied extensively. Witebsky *et al.* (128) prepared a CF antigen by heating an extract of larvae in a boiling-water bath. Melcher (79) prepared acid-soluble and -insoluble fractions from an extract of delipidizing lyophilized larvae. Labzoffsky *et al.* (70) isolated eight fractions from larvae with a pyrimidine extraction. Chemical analysis revealed glycoprotein and carbohydrate characteristics. The antigens reacted differently to circulating antibody in the serum of rabbits at different stages of the infection. Sleeman and Muschel (106) fractionated the larval antigen into ethanol-soluble and -insoluble components. Of interest is the fact that Witebsky used his boiled antigen at two dilutions (1:2 and 1:20) for maximum sensitivity in the CF test. These dilutions corresponded to Sleeman and Muschel's fractions with regard to serologic reactivity. The ethanol-soluble antigen absorbed S. *typhosa* agglutinins present in the sera of trichinella patients. Chemical analysis for these antigens (105) revealed that the ethanol-soluble antigen was a glycoprotein (75 per cent protein and 15 per cent carbohydrate), with the carbohydrate portion composed of only glucose units. In the light of Weiner and Neely's (125) studies, one would expect to find some rhamnose as well. Attempts to split off the protein or the carbohydrate resulted in denaturing the antigen. The ethanol-insoluble antigen was a nucleoprotein, with the nucleic-acid portion containing DNA (60 per cent) and protein (14 per cent). The protein was the antigenic substance in the complex.

Tanner and Gregory (121) analyzed extracts of larvae of *T. spiralis* by immunoelectrophoresis (IE). Tanner (119) found that while most of the trichina antigens were proteins that could be precipitated with 5 per cent trichloracetic acid, the major antigen could not be so precipitated and contained some polysaccharides. This component had an isoelectric point similar to human gamma globulin and was heat-labile. Enzyme susceptibility studies (120) identified it as a mucoprotein. The specific enzyme employed to degrade this antigen was mucoprotenase lysozyme.

The antigens of *Echinococcus* species (hydatid fluid, scolices, and membranes) have been popular materials for antigenic analysis. We chose hydatid fluid of *E. granulosus* early in our antigenic analysis work because it was a biological fluid with a strong antigenicity and bore a striking resemblance to paper electrophoretic patterns obtained with serum of the host (42). To date we have identified 19 antigenic components in sheep hydatid fluid (24). At least two polysaccharides have been described (2, 64), as have end products of carbohydrates and protein metabolism (1).

Polysaccharide antigens have been isolated from laminated membrane and probably germinal membrane by a number of workers. Agosin *et al.* (2) separated the polysaccharide antigens in two components by electrophoresis and found a mobility similar to that of glycogen. The second contained glucosamine and galactose. Kilejian *et al.* (64) isolated a mucopolysaccharide. Working in our laboratory, she was able to coat latex particles with this

antigen and found it to be reactive with sera from immunized animals but not with sera from infection. Magath (77) reported that an echinococcus antigen reactive in the CF test moved like a gamma globulin by immunoelectrophoresis. Paulete-Venrell *et al.* (90) reported that their antigen moved in an immunoelectrophoretic field like beta and gamma globulins. Harari *et al.* (44) identified an immunologically active component in hydatid fluid as a globulinoid protein. Glycolipid and glycoprotein have been identified by Pautrizel and Sarrean (91) in hydatid fluid antigens. The antigens of *Echinococcus* were recently reviewed by Kagan and Agosin (51).

Gel-diffusion and IE analyses of helminth antigens

The characterization of parasitic antigens by the various gel-diffusion methods has elucidated their complexity and has provided a useful assay for their purification. The techniques are relatively simple and do not require elaborate equipment. They have certain limitations: the number of lines observed in an agar gel precipitin test represents minimum numbers of antigenic components that are at equivalence. It is therefore important to evaluate several dilutions of antigen, or more rarely of antiserum, for the maximum development of antigenic complexes. The introduction of radiolabeled parasite antigens has extended the usefulness of this technique in parasitologic studies (34).

The strength of the gel-diffusion test is usually limited by the antibody content of the antisera employed. Antisera prepared in rabbits against a number of helminth worms in our laboratory were made by injecting rabbits with 2 mg of lyophilized antigen suspended in 0.5 ml of saline with an equal amount of complete Freund's adjuvant. A rabbit received six injections over a three-week period, or a total of 12 mg of antigen. We thought we were injecting large doses of antigen, but Biguet and Capron use 20 mg of antigen per inoculation (14). The antisera they employ after six months or a year of immunization contain many more antibodies to major and trace components in the antigens they assayed. It is for this reason that Biguet *et al.* (19) reported so many cross-reactions between cestodes, helminths, and nematode species. The differentiation of closely related species is also difficult with such composite antisera (13).

At least four antigens common to adult *S. mansoni* and the laboratory mouse host were demonstrated by Damian (28). In addition, a Forssman hemolysin was demonstrated in rabbit anti-schistosome sera. Analyses of the various stages of the schistosome life cycle were made by Capron *et al.* (20). These workers were able to demonstrate 21 antigens in extracts from adult worms, 11 shared by adult and egg, 14 with cercariae, and 12 with excretions and secretious products. There were four bands common to the parasite and the hamster host and five common to the parasite and the snail host *(Australorbis glabratus)*. By labeling extracts of *S. mansoni* with I^{131}, Dusanic and Lewert (34) were able to differentiate five or six antigen-antibody complexes by cellulose acetate electrophoresis, as contrasted to two to five lines demonstrable in agar gel precipitin tests with the same sera.

Capron *et al.* (22) reviewed their work on gel-diffusion analysis of *S. haematobium*, *S. japonicum*, and *S. mansoni* that had been completed since 1962. They were able to find 19 of 21 immunoelectrophoretic fractions of *S. mansoni* common to *S. haematobium* and ten antigens common to *S. japonicum*. Analysis of a large number of sera from infected individuals indicated at least nine precipitin bands in serum from patients with schistosomiasis mansoni, six in schistosomiasis haematobium and seven in schistosomiasis japonicum. In experimental schistosomiasis mansoni these workers found 18 antiadult, ten anticercarial, and at least ten anti-egg precipitins. Similar immunodiffusion studies of schistosome antigen were made by Damian (27) and Sadun *et al.* (94). Dodin *et al.* (32) found six to eight precipitin bands by the Ouchterlony and IE technique in sera of patients under treatment. Of great interest was the fact that they could

visualize circulating antigen on the seventh day of treatment in the serum of these patients. This antigen migrated toward the anodic side of the reaction. Kronman (68) analyzed a cercarial extract of S. *mansoni* and was able to resolve it into three components by DEAE cellulose chromatography. Peak 1 moved 35 mm anodically and reacted with human antisera; peak 2, 22 mm; and peak 3, 14 mm. The latter two components were not active in diagnostic tests.

Caetano da Silva and Guimarães Ferri (17) found one to four precipitin bands in the serum of 78 per cent of patients with hepatosplenic schistosomiasis, as against one band in only 38 per cent of patients with hepatointestinal schistosomiasis. In a second paper (18) these authors published data on a reverse immunoelectrophoretic technique. Serum was fractionated in an electrical field and developed with antigen of S. *mansoni*. Precipitin bands in the IgM and IgG position were visualized.

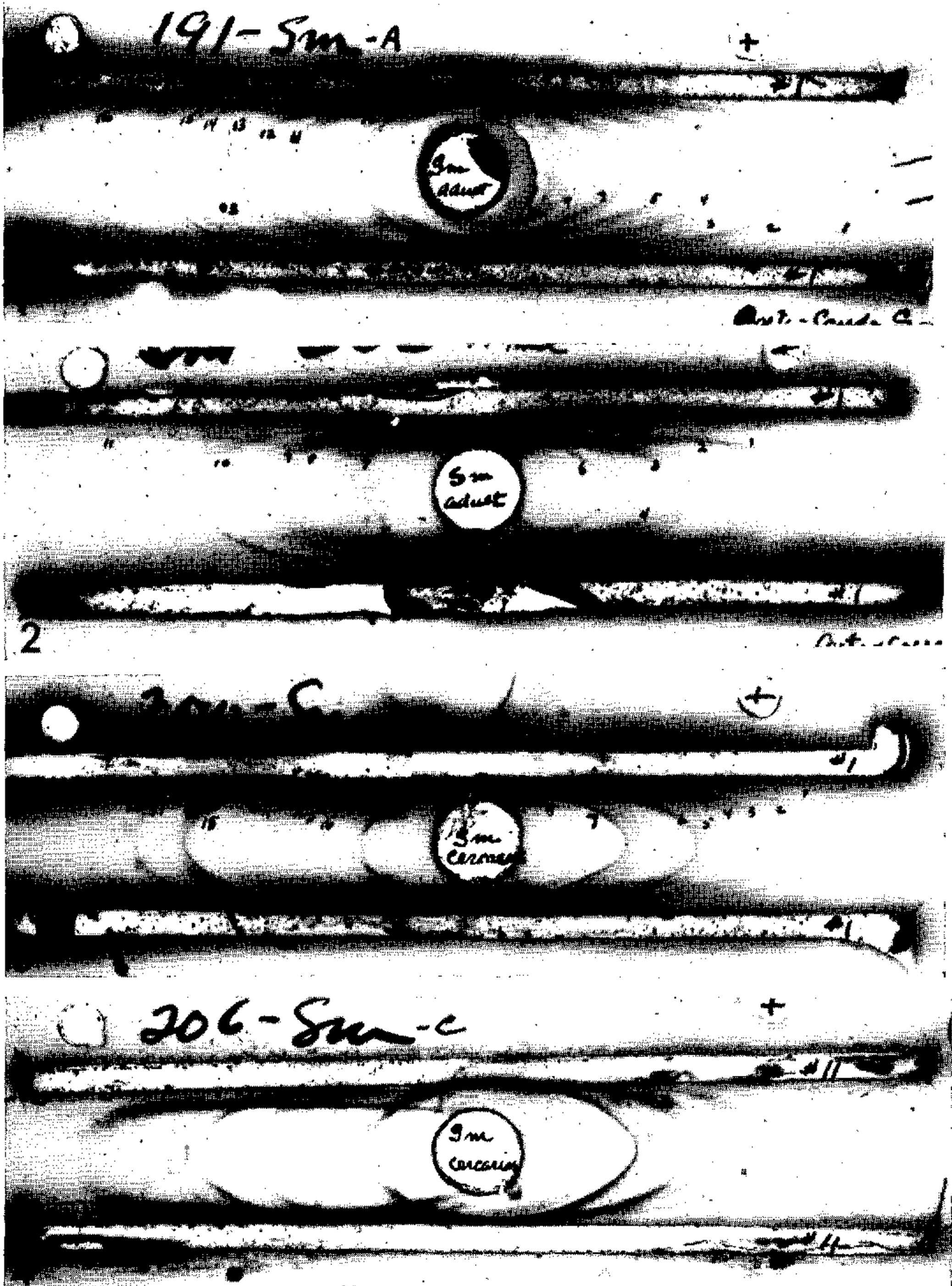
Kent (63) analyzed adult and cercarial extracts in terms of protein, carbohydrate, and lipid. He was able to show that a considerable portion of the lyophilized antigen is water-soluble. Ten protein systems in adult and eight in cercariae were detected by immunoelectrophoresis. One cross-reacting antigen with *T. spiralis* was demonstrated. Biguet *et al.* (10) were able to demonstrate eight proteins, five glycoproteins, and one lipoprotein in adult extracts of S. *mansoni*. Stahl *et al.* (116) were able to demonstrate antibodies to egg antigen-antibody complexes.

In our work (53) we were able to demonstrate by agar gel analysis seven specific adult worm, three cercarial, and five egg antigens. In all, 25 different antigenic bands were demonstrated by Ouchterlony gel-diffusion analysis. Analyses were made of antigens prepared by various methods such as delipidization with anhydrous ether (Chaffee antigen), petroleum ether (Melcher antigen), and crude extract. In these extracts, five of seven adult antigens were shared. Immunoelectrophoretic studies with antisera prepared in rabbits showed the complexity of our schistosome extracts. An extract of adult S. *mansoni* containing 0.87 mg N/ml was developed after electrophoresis with a serum prepared against the crude antigen (Figure 1).* An extract of adult worms prepared by the Melcher (79) technique was developed with the same serum (Figure 2). At least 16 components in the crude extract and 11 components in the Melcher extract were identified. A delipidized cercarial extract (prepared by extraction with anhydrous ether) developed against the same antiserum revealed at least 18 components (Figure 3). The same antigen developed with an anti-Chaffee adult serum shows a slightly different configuration (Figure 4). Absorption studies indicated that all but perhaps one band are shared by the cercariae and the adult.

An immunoelectrophoretic analysis of *F. hepatica* antigen by Biguet *et al.* (11) revealed 7 protein fractions, 2 glycoproteins, and 6 lipoproteins. Of 15 fractions visualized with rabbit antisera, 5 were specific. Szaflarski *et al.* (117) attempted to characterize an antigenic mucoprotein prepared with sulphosalicylic acid using papain and rivanol without success. Capron *et al.* (21) identified C reactive protein in extracts of *F. hepatica* as well in a number of other helminth parasites (12).

Tanner and Gregory (121) showed in their gel-diffusion studies on extracts of larvae *T. spiralis* that of the 11 bands they identified individual rabbits developed antibodies to only some. They also compared the crude extract of larvae and a Melcher-type larval antigen. In most instances they found that the differences between a crude buffered saline extract and antigens prepared by alkaline and acid extraction after delipidization (Melcher-type) were quantitative, not qualitative. Dymowska *et al.* (35) fractionated larvae of *T. spiralis* on a starch block and assayed 26 protein fractions. Among these fractions, 9 to 14 proved to be serologically active. They contained acid phosphatase and hyaluronidase. The antigenic structure of *T. spiralis* was analyzed in detail by

* In each figure the numbers designated to a line of precipitation are based on order of appearance and not antigenic relationship.



An immunoelectrophoretic analysis of adult and cercarial antigens of *Schistosoma mansoni*. FIGURE 1. The antigen is a crude extract of adult worms of *S. mansoni* developed with a homologous antiserum prepared in rabbits. FIGURE 2. A delipidized (Chaffee) extract of adult *S. mansoni* developed with an antiserum against the crude worm extract. FIGURE 3. A delipidized extract (Chaffee) developed with the crude adult worm antiserum. FIGURE 4. A delipidized cercarial extract (Chaffee) developed with a homologous rabbit antiserum.

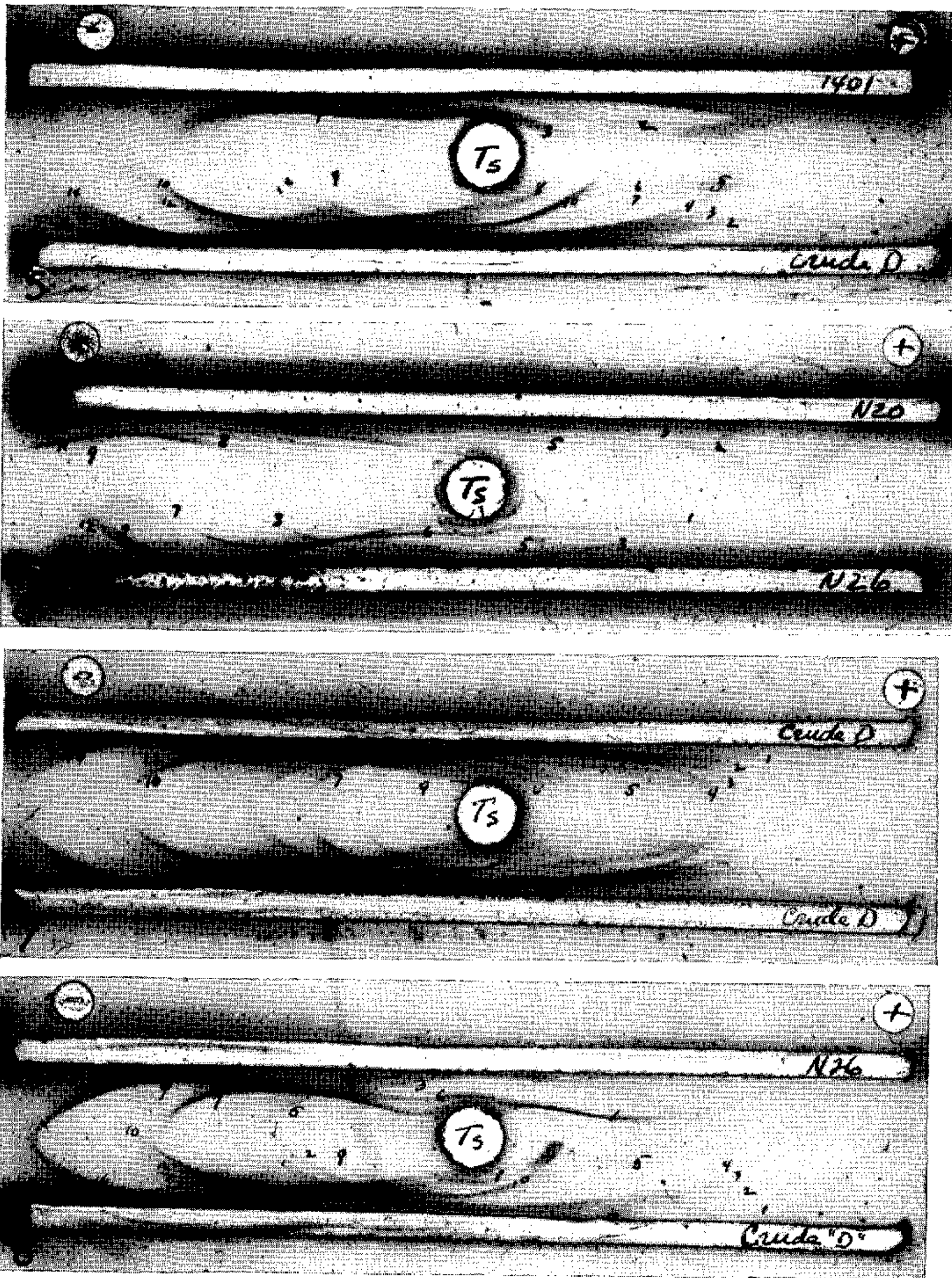
Biguet *et al.* (14). With antisera produced by immunization in rabbits, 19 antigenic components were identified; with antisera from infected rabbits, 10 bands. The appearance of antibodies in the serum during the course of infection was also studied. The total number of 19 antigenic components is reached after five weeks of immunization in rabbits. Recent studies on the specificity of *T. spiralis* antigens were made by Lupasco *et al.* (72), Moore (80), and Dusanic (33).

In our own immunoelectrophoretic studies with a larval antigen of *T. spiralis* prepared by the Melcher technique (79) containing 2.34 mg N/ml, we identified 12 bands in serum from infected rabbits, 5 bands in human diagnostic serum, 11 bands with an antiserum prepared against a metabolic antigen, and 16 bands with an immunized-rabbit antiserum. The reaction of this antigen developed after electrophoresis with a human diagnostic serum (1401) and with a rabbit immunized antiserum (crude D) shows the antibody complexity of these sera and a lack of identity in the bands, which were visualized since they did not join after three days of incubation (Figure 5). In Figure 6 the antigenic development of this antigen with the sera of two infected rabbits is depicted. Note the difference in the patterns developed on the cathodic portion of the reaction. Figure 7 shows antigen developed with an antiserum prepared against a crude larval antigen; after three days of incubation, common antigenic components joined and coalesced. In Figure 8 an infected rabbit serum (N26) and a rabbit antiserum (crude D) were used to develop the reaction to detect common components in these bleedings; only two or three antigen-antibody bands were shared. In Figure 9 a human diagnostic serum (1401) and an infected rabbit serum (N26) were used to develop the antigenic pattern; only bands 4 and 7 are common. In Figure 10 a similarity in pattern may be noted between the infected rabbit serum above (N20) and an immune rabbit serum (LXS) prepared against metabolic secretions of larvae (LXS antigen). In Figure 11 the LXS antiserum is compared to the crude larvae antiserum with very little evidence for antigenic sharing of components. In Figure 12 the LXS antiserum is compared to a human diagnostic serum and one component appears to be shared. The antigenic complexity of a delipidized extract of larvae of *T. spiralis* as revealed by this type of analysis is very great. In all the reactions, only a few components are clearly shared—the remainder may be different.

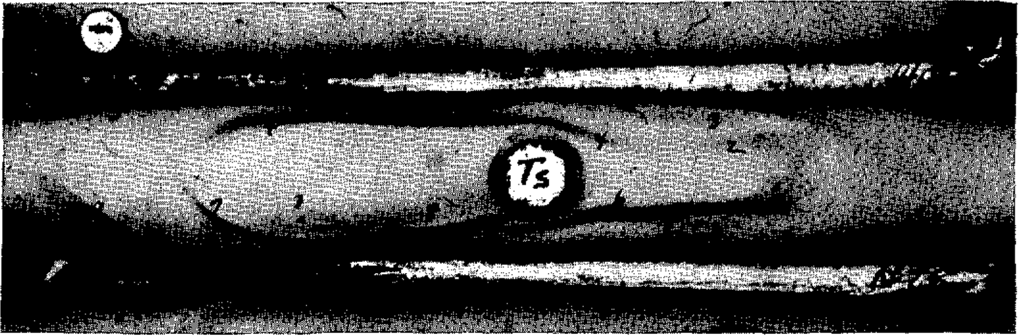
Agar gel analysis of *Ascaris* tissues and extracts were reported by Kagan (48), Kagan *et al.* (56), Soulsby (109), and Huntley and Moreland (46). Tormo and Chordi (123) prepared polysaccharide and protein extracts of *A. suum* for analysis by immunoelectrophoresis. A total of 20 antigenic components were visualized by their antisera. Of this group only seven antigenic components were found in sera of infected animals and natural infections in man.

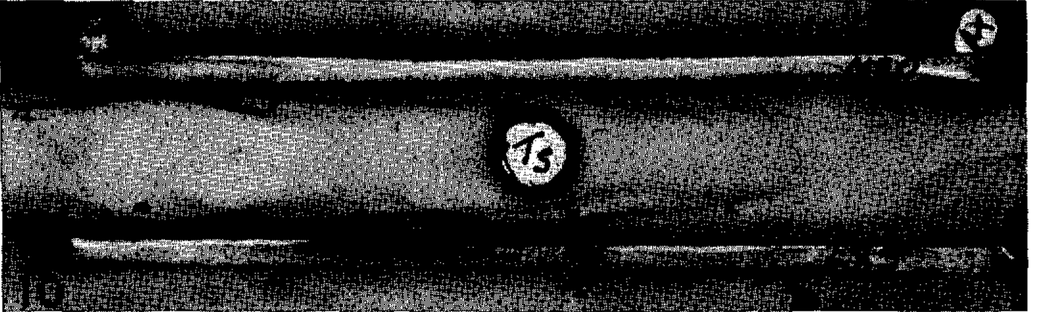
Our studies on analysis of *E. granulosus* hydatid fluid and extracts of cysts of *E. multilocularis* have been reviewed in several publications (49, 53). We found only 9 of 19 components in hydatid fluid to be of parasitic origin. We were able to isolate gamma globulin and albumin antigen that gave lines of identity with serum gamma globulin and serum albumin of the host (54). With the technique of right-angle agar gel analysis, diffusion coefficients of our diagnostic antigens were measured (4). When antigen and antibody at equivalence are allowed to diffuse from troughs cut at right angles in an agar plate, a narrow line of precipitate is formed. The tangent of the angle made by this line with the antigen trough is equal to the square root of the ratio of the diffusion coefficients of antigen and antibody. When rabbit or human antibody is used the diffusion coefficient of the test antigen can be calculated. Values from 3.2 to 7.2 x 10^{-7} cm^2/sec. were obtained for seven hydatid fluid components tested with a rabbit antiserum. Three of four parasitic components in an analysis of human sera had diffusion coefficients of 1.6, 1.7, and 2.0 x 10^{-7} cm^2/sec. The diffusion coefficient data suggest molecular weights close to one million for these diagnostic antigens (3).

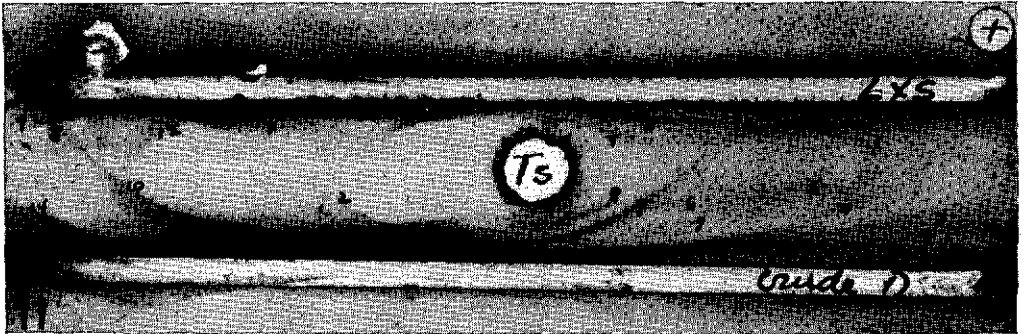
Our recent studies on chromatographic

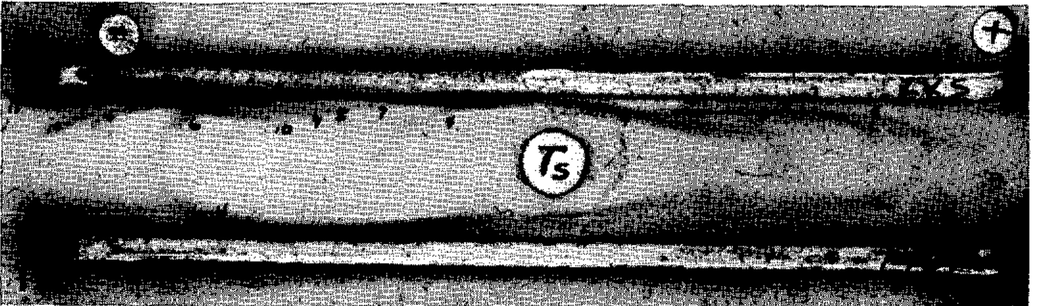


An immunoelectrophoretic analysis of a Melcher extract of larvae of *Trichinella spiralis.* FIGURE 5. The larval antigen developed with a human diagnostic serum (1401) above, and a rabbit antiserum prepared against a saline extract of larvae. FIGURE 6. The larval antigen developed with the sera of two rabbits with experimental infections with *T. spiralis.* FIGURE 7. The larval antigen developed with an antiserum against a saline extract of larvae of *T. spiralis.* FIGURE 8. The larval antigen developed with an infected rabbit serum (N26) above, and an immunized serum (crude D) below.









An immunoelectrophoretic analysis of a Melcher extract of larvae of *Trichinella spiralis.* FIGURE 9. The larval antigen developed with a human diagnostic serum (1401) above, and an experimental infection in the rabbit (N26) below. FIGURE 10. The larval antigen developed with an experimental rabbit infection serum (N20) above, and an antiserum prepared in rabbits against a metabolic secretions and excretions antigen (LXS). FIGURE 11. The larval antigen developed with the metabolic secretions and excretions antiserum (LXS) and a crude larval antiserum (crude D) below. FIGURE 12. The larval antigen developed with the LXS antiserum above, and a human diagnostic serum (1401) below.

separation of diagnostic antigens emphasize the importance of gel-diffusion assay. Although column chromatograph of hydatid fluid of *E. granulosus* and *E. multilocularis* (18, 82) appeared to separate host from parasite components, agar gel analysis indicated that complete separation of the two groups did not take place, since molecular screening techniques cannot separate many α_1 and α_2 globulin-like antigens of host origin that migrate with similar parasite antigens.

Protozoan antigens

Antigenically reactive polysaccharides have been isolated from *Trypanosoma cruzi* (41). Fife and Kent (36) separated protein and polysaccharide components from *T. cruzi* and evaluated their sensitivity and specificity in the CF test. The fractionated antigens were more specific than the crude extract but less sensitive. The protein component was the best and most economical antigen to use. Von Brand (15) reviewed the information on the chemical composition of *T. cruzi*. Exoantigens or secretory antigens produced by *T. cruzi* have been studied and a glycoprotein has been described (122).

The chemical composition of an African trypanosome was studied by Williamson and Brown (126) and Brown and Williamson (16).

The *Leishmania* organisms must share a common antigen with mycobacteria, since the latter have been used by a number of workers in South America as a diagnostic antigen in the CF test for leishmaniasis. This antigen, however, could not be isolated or characterized in gel-diffusion studies (52).

A number of protozoan species have been studied by agar gel and immunoelectrophoretic analysis. Krupp (69) recently evaluated 11 amebic antigens by IE, and similarities between some strains of *Entamoeba histolytica* with high and low pathogenicity was observed. Goldman and Siddique (40) analyzed two substrains of *E. histolytica* and showed some antigenic disparity.

The studies of Schneider and Hertog (101) on 16 strains of *Leishmania* indicated that two immunologic groups of human leishmaniasis were present in Panama with wide geographic distribution. García (37) showed that *L. tropica* has three heat-labile and one heat-stabile component.

Nussenzweig *et al.* (85) separated a number of *T. cruzi* strains into three antigenic groupings by means of agglutination and precipitin tests in agar. Both type- and group-specific substances in groups A and B were reacted. In a further analysis (84) 23 strains were studied and it was shown that most human strains are Type A but some were of Type B.

Antigenic analyses of plasmodia made by immunoelectrophoresis and agar gel by Spira and Zuckerman (114) revealed seven components in extracts of *P. vinckei*. Zuckerman (133) compared *P. vinckei* and *P. berghei*, and found several common antigenic components. Using polyacrylamide gels, Sodeman and Meuwissen (108) found at least 21 bands in *P. berghei* extracts. From 3 to 12 participating antigens have also been described in plasmodia extracts (7, 8, 29, 103, 25). Chavin (23) found 10 to 15 bands in extracts of *P. berghei* in polyacrylamide gel, 4 to 7 lines on IE, and 8 to 10 lines by double diffusion in tubes. An interesting aspect of Chavin's work was the presence of all the bands in IE on the anodic side of the electrical field. Mouse-hemoglobin protein comprised a significant portion of the extract. The parasite components had electrophoretic mobility in the beta-to-albumin range and could not be separated from the host components. Similar difficulties are reported in our hydatid-fluid fractionations in separating host and parasite components by ion-exchange chromatography (81, 82). Spira and Zuckerman (115) have extended the analysis of plasmodia species by disc electrophoresis for seven plasmodia species. Differences were apparent between all the species, and their chemical complexity is evident from the large number of components developed in their preparations.

Comment

This review is far from complete, and many excellent publications on the analysis of para-

sitic components in the immunology and serology of paragonimiasis (132, 47, 96, 97, 131) and filariasis (118, 95) and other parasites of veterinary (6) and medical importance have been omitted. Studies on the fractionation and characterization of parasitic materials are at a crucial stage of development. It is important to characterize the antigenic complexity of our diagnostic and immunogenic materials. Research must be focused, however, on characterization of the specific immunological components. To accomplish this end we need a stronger biochemical approach. We have to staff our laboratories with scientists capable of working with the enzymes and chemical components that interact in our immunologic reactions. We need personnel who can use the complex preparative chemical techniques, such as preparative column electrophoresis, gas chromatography, and other tools emerging from immunologic research in related fields.

Parasitic materials are excellent sources for both applied and basic immunologic studies. The successful parasite has solved the host's "graft-rejection" response. The self-not-self problem in immunology might be as fruitfully studied with a host-parasite system as with the graft-rejection system.

Finally, we need specific antigens for parasitic vaccines and diagnostic tests. Some hosts develop strong functional immunity against their parasites. Our feeble attempts to stimulate this immunity by vaccination have been far from successful in most parasitic infections. Once the immunogenic substances have been characterized and synthesized, practical vaccines for parasitic infections will be available. Parasitic immunodiagnosis will be greatly enhanced when the laboratory can prepare specific and active diagnostic reagents. The infected hosts develop a large number of antibodies. Analysis of these antibodies and the development of specific antigens for their detection are the challenges of the future.

Moderator: The discussion of these topics will be introduced by Dr. Finger.

DISCUSSION

Irving Finger

It may seem a little unusual for a geneticist who studies a completely nonparasitic organism to be speaking at such a session, but I hope the relevance of our studies to those described by the two previous speakers will become apparent.

We have studied primarily the genetics and regulation of antigen synthesis in *Paramecium aurelia*. It is obvious that the major advantage possessed by free-living protozoa for studies on these topics lies in the ability to make controlled matings and therefore to analyze the relative importance of genetic and nongenetic determinants in the control of a particular trait. Similarly, cytoplasmic as contrasted with nuclear contributions to the determination of a character can be distinguished. The other primary benefit to be derived from studies of free-living forms is, of course, that the environment can be more carefully controlled and it may be possible to analyze the various contributors in that environment that determine antigenic composition.

I should like to describe briefly some of the characteristics of the surface antigens of paramecia and the genetics and regulation of their synthesis, particularly as these bear on the topics discussed by the previous speakers. Every cell can express at least a dozen surface antigens. These antigens are generally recognized by a cell's immobilization in a specific antiserum prepared against particular serotypes. Even though a cell has the ability to make a multiplicity of antigens, generally only one antigen is expressed at a time—which one being determined by a complex interaction of nucleus, cytoplasm, and environment.

The antigens themselves are proteins with a molecular weight of about 310,000. They are probably not made on the surface but migrate to the surface and ultimately to the cilia. The most disturbing thing about these antigens, as far as the experimenter is concerned, is that their function is completely unknown. A cell apparently has no particular selective advantage in possessing one antigen rather than another. Apart from the phenomenon of mutual exclusion—the expression of but one antigen at a time—the striking feature of these antigens is that one antigenic type can easily be transformed into another. This transformation is readily reversed and is not due to mutation.

From this brief summary, I should like to turn to more specific features that are relevant to the antigens of parasites. When antigenic types transform to each other, they often transform to unrelated antigenic types. But even if two antigens are serologically quite distinct, they appear still to possess the same "function" and to be remarkably similar in their chemical features. It is obvious that this transformation process, and the fact that the antigens may be immunologically distinct, will have a great bearing on assigning a particular function to a class of antigens, since one of the criteria for correlating function with a molecule is whether this function disappears when the molecule disappears. Here, however, the function of a class of molecules persists even when the original specific molecule no longer exists.

Secondly, the antigens that can be detected

on the surface are not necessarily the only ciliary antigens that the cell possesses. By this I mean that there may be primary antigens that are recognized by the immobilization test and can definitely be stated to be external, and there may be secondary immobilization antigens that are not recognized by immobilization tests but can be detected when the cell is broken open and all the antigens are extracted.

Therefore, in a sense, the phenomenon of mutual exclusion that I mentioned earlier is not necessarily an all-or-none phenomenon; what may be excluded on the surface may not be excluded internally. These secondary antigens, obviously, may have potential significance by eliciting the formation of antibodies, especially if there is lysis of cells within the organism.

A third point of relevance, particularly to the work described by Dr. Brown, is that the environmental influences that determine the particular types a paramecium will possess at any time are manifold and include such things as antiserum, temperature, cultural conditions, enzymes, temperature shock, inert proteins, and stage in the life cycle.

Another feature of the antigens of paramecium that is of possible significance to studies with parasitic protozoa is the recent finding in our laboratory that the various serotypes release into their medium material that can induce transformation when added to other serotypes. The material released does not act in a nonspecific fashion. Different media have propensities for inducing different kinds of transformations. Our studies thus far suggest that the substances put out into the media are not related to the antigens and act by specifically repressing the activity of certain genes.

I should like now to discuss briefly the effect of serum on cells. Not only does immune serum induce transformation, but normal serum from an unimmunized animal (in our case, the rabbit) may have a very profound effect on the kind of antigen expressed by a cell. The factor in serum that has this effect is present in the 0-40 per cent ammonium sulfate cut of the serum, the same fraction that contains the immunoglobulins. The orginal cell is not visibly affected by such serum, but the serotypes of its descendants are altered.

With regard to immune serum, there are several features of interest here. It might be expected that, when antigen is found on the surface, immobilizing antibody would induce transformation by binding this surface antigen. We have found, however, that this is not necessarily the case. Rather, it seems more likely that the antibodies against surface antigens only influence the transformation of antigenic type if that antigen is being actively synthesized. It is probable that antibodies do not act through tying up the surface antigens but probably penetrate the surface of the cell and act at the synthetic sites, or conceivably the sites where the secondary antigens are located.

We have also been able to show that antisera that are prepared against media in which paramecia have been living and that possess no immobilizing titer can markedly influence the pattern of antigen synthesis. Apparently what is found in these sera is antibodies directed against the substances released by the paramecia that in turn repress gene activity.

So we see that the effect of a serum can be quite complex. It may contain nonimmune substances that can induce antigenic transformation and perhaps allow the descendants of the original cell to escape the effect of specific antibodies. Antiserum can affect the synthesis of antigens that are not on the surface; and, finally, antibodies against substances other than the antigens themselves, substances that control the synthesis of the antigens, may have a marked effect on transformation.

Another thing that is pertinent to some of the topics Dr. Brown discussed is that we have found no antibodies except those against the ciliary antigens lethal to the paramecia. There appears to be no general antigenic substance that when combined with antibody causes the death of the cell.

Let me conclude with a few remarks about the gel-diffusion techniques that have been extensively used by Dr. Kagan and many others in the study of the antigenic mosaics

that make up cells. There are several advantages to analysis by means of gel-diffusion techniques compared with the more classical precipitin tests, hemagglutination, complement fixation, and so on. For example, a purified antigen is not needed for unambiguous results; multiple mixtures may be studied and roughly quantitated. The major difficulty with the gel-diffusion techniques—including those using tubes, such as Preer's and Oudin's, and those using flat surfaces, such as Ouchterlony's and immunoelectrophoresis—is that there is an alarming tendency for artifacts to appear. The possibility of these artifacts must be very carefully controlled for. However, once these are taken into account, the Preer technique, which is the most sensitive of those I have mentioned, may be especially useful in following the isolation of antigenic components and for diagnostic purposes, as Dr. Kagan has shown. In fact, the Preer technique can be used (by following a modification of the Oudin profile method) to make qualitative comparisons of various antigens with a several-fold increase in sensitivity.

A problem with gel-diffusion techniques is that they are much too easy to use. Anyone can set up gel-diffusion tests from scratch in an afternoon and have data the following day. It is the interpretation that takes a great deal of care and experience and is not always easy. Reactions of identity in Ouchterlonys must always be considered tentative, depending on the serum. Reactions of partial identity are only convincing when accompanied by absorption experiments.

Lastly, I want to emphasize, along with Dr. Kagan, that the determination of the number of antigens in a particular preparation must always take into account the possibility of band splitting and the question of whether one is in antibody- or antigen-excess regions of the precipitin curve.

Moderator: I want to thank you particularly, Dr. Finger, for introducing the concepts derived from the studies on the genetics of paramecia. This knowledge is so fundamental for immunoparasitologists that I think we should discuss it thoroughly. As I understand it, the production of antigens by the paramecium depends, if I recall my genetic studies, on the genes from the macronucleus as well as on the cytoplasm and also on environmental factors.

Finger: Yes. The environment determines the particular cytoplasmic state, which determines which gene in the macronucleus will work.

Moderator: I am not very clear about the mechanism of mutual exclusion; I believe it is a rather complex matter.

Finger: No one is clear about that mechanism. In fact, that is the subject of our research.

Moderator: One of the things I want to ask is how you would interpret the stabilizing effect of the vector mentioned by Dr. Brown. You remember that he called attention to the fact that the vector had a stabilizing effect on the antigenic variants of trypanosomes produced in the course of infection, so that the parental type was obtained when it got back into the vertebrate.

Finger: Offhand I cannot think of a hypothesis, because of the apparent nonspecificity of the phenomenon. We are carrying out work based on a hypothesis that invokes the persistence of an antigen due to the feedback of the antigen itself on its synthesis. But I do not know what the factor might be in Dr. Brown's vector for maintaining a particular parental type.

Brown: There was the point that when the parasite passes through the vector, it changes structurally and the electrophoretic pattern of its cytoplasmic proteins also changes. In particular, the 4S group, which is the major group of variable antigens, appears to be much less evident in the insect form. It is apparently repressed there. But why, when it gets back into the vertebrate, the parasite should develop as the parent antigenic type, I do not know. This reversion is not always complete. Often traces of the original variants come through along with the parental type.

Finger: Do you know whether this could be due to selection in a mixed population?

Brown: It could be, yes. I have perhaps oversimplified slightly: when I say there is a succession of populations, I mean that there is a succession of population mixtures, particularly late in the infection. I have in fact isolated a parent form from such a mixture quite late in the infection, so it is possible that this is the one that is selected and that comes through the vector.

Finger: With reference to the paramecium situation, there is no selection among the paramecium serotypes. It is a conversion of a population of one type to a new population.

I wonder if you would expand on what I found to be of particular interest—the conversion of the crithidial to the trypanosome to the leishmania type by increasing the concentration of an anticrithidial serum.

Brown: Unfortunately, there is not much more to say about that. This work was done a few years ago by Professor Adler. He prepared the rabbit anticrithidial serum and incubated his crithidia in it and in that way was able to show that a single crithidium produced a colony of either trypanosome or leishmanoid forms, depending on the serum concentration. This work has not been continued.

Finger: The crithidium then shares antigens with trypanosomes, and these antibodies are perhaps responsible for the conversion?

Brown: They do share some antigens, and one would think that the difference may perhaps be quantitative rather than qualitative.

Finger: That is right.

Moderator: I think this illustrates the need for immunologists and geneticists to get together and study certain fundamental problems.

I was very much interested in what Dr. Brown said about the hemagglutination reaction's depending on the red cell membrane. Do you think it is some antigen diffusing from the parasite that sensitizes the red cells or modifies a protein of the red cell and produces this? And, just to ask all the question at the same time, if complement is added, do the red cells lyse or not? Is the phenomenon related to passive hemagglutination and passive hemolysis?

Brown: In reply to the first question, it is interesting that the red cell infected with the immature parasite, the trophozoite, does not agglutinate. It is only when the parasite matures and forms the schizont that it will do so. At this stage, the surrounding red cell itself is breaking down. The membrane is visible in EM sections, but the contents are pretty much destroyed, and I imagine—although there is no evidence for this—that the parasite antigen can then get into the red cell membrane. If the red cell membrane is removed, these naked parasites will not agglutinate in immune serum. This may be because the removal of the red cell membrane removes a lot of the antigen, or perhaps the complete antigen involves a parasite component and a red cell component. We have in fact shown that if the red cell is lysed with anti-red-cell serum, a certain amount of parasite material is released and can be collected in the lysate formed.

As for your second question, if complement is added to schizont defective cells in immune serum, they will not lyse. The reason for this, we think, is probably that the red cell, being so empty, is more or less lysed anyway. Even if the complement punches holes in the red cell membrane, there will be no osmotic effect to burst the cell.

Goodman: Along the same line as Dr. Bier's questions on your very interesting observation about the hemagglutination of the red cell having something to do with the red cell membrane, and perhaps with associated antigen from the malaria parasite, is this agglutination only of the red cells with the schizonts in them? Does the agglutination involve red cells that are not parasitized? Have you been able to find any factor you can put on a normal cell, let us say, that would cause it to be agglutinated? Those are my specific questions.

On a more general level, there have been some philosophical interpretations of the destruction of red cells in malaria in which autoimmune responses have been implicated in theory. It sounds as if this would be rather unlikely in the light of your observations.

Brown: With regard to the first point, in our system normal red cells are not agglutinated at all; they are completely distinct. And, as I said earlier, the cells containing the immature parasite do not agglutinate either. So the reaction of the schizont-infected cell is quite distinct.

Goodman: When you do the agglutination test, are 100 per cent of your red cells parasitized so that they all agglutinate, or do you just see agglutination of those that have the mature schizonts? Is, for example, how you do the test affected?

Brown: We have done both. Normally and routinely for the test, we collect the schizont-infected cells, but we have also tested mixtures; in fact, we have also transfused immune serum into monkeys containing infected and noninfected cells and shown that when the schizont-infected cells are put onto a warm stage they will then agglutinate and the normal cells will stay quite separate.

Your question about autoimmune responses raises, I think, a very debatable point. I think the lysis of normal red cells could be explained in quite a number of ways. One thing we are looking into at Mill Hill is the possibility that cysfaccinic(?) acid, which is present in the parasite and is quite a strong lytic agent, may be responsible. I think it can be explained in biochemical terms just as easily as in immunological ones.

Cohen: My comment is directed to Dr. Finger, but it bears on the earlier presentations. One can not help being impressed with the apparent multiplicity of proteins during the various stages of differentiation of these intermediate forms. I feel inclined to wonder, as a biochemist, whether the entities are too crude for determining whether they are in fact genetically different structures. We have learned, of course, that it is the subunits of the proteins that are genetically controlled, and it would seem to me that perhaps the important thing is to devote some effort to characterizing a few good proteins and finding out the nature of their polymeric and other tendencies to aggregate. Reference was made to their relative ability to form glyco and other proteins. I do not know whether these have covalent bonds or whether they are merely adducts of some kind, but it would seem to me that before the genetic implications in going from one stage to another can really be analyzed, a subunit structure for identification ought to be settled on. We certainly know that many proteins in their subunit form will have one kind of immune specificity but in the aggregate form may be blocked because of the specific groups that are inducing the antibody formation. While this may only complicate the story, it does seem that we should be rather cautious at this stage in taking the apparent complexity too literally. There may be a simplicity if we can get to the subunit stage.

Finger: With reference to paramecium, I can answer in this way: Unfortunately, the situation is as complex as I presented it. The proteins have been purified and characterized chemically and physically. They are made up of nine subunits, apparently arranged in sets of three trimers, and each distinct antigenic type is controlled by a different locus. The genetic work that has been done shows that these loci are unlinked. We know that the various forms of antigenic types I have described, although they have related amino acid compositions and sedimentation coefficients, also have differences. Fingerprints have been made. It may be a little unfair to talk about the paramecium antigens, because we have been fortunate. Dr. Preer and Dr. Steers have purified them sufficiently for actual analysis. In this case, therefore, I do not believe the question of one's being a polymer of another is applicable. In fact, we have even studied complementation and hybrid forms of the antigens. I do think that, as Dr. Kagan indicated, in the parasitic forms there is greater complexity; I am delighted to be working with paramecium.

Borsos: Transformation of cells may be the result of the action of an antibody on the cell surface. However, there are other substances—such as phytohemagglutinins—that can induce lymphocytic transformation, that can induce biochemical changes inside the cell

and stimulate synthesis of DNA, proteins, and cell divisions. Has anything like phytohemagglutinin been tried on parasites of this sort to induce changes in the antigens?

Another point I should like to make is that antibodies, as far as we can tell, do not kill. They may occupy sites and the result of the occupation may be a steric hindrance of some sort, but there seems to be no biochemical activity involved in antibody interaction with antigens. It is therefore not surprising that antibodies will not kill a parasite. Perhaps occasionally a miracidium may be blocked in its movement. But other substances, such as complement, are needed to make an antibody cytocidal.

Furthermore, not all classes of antibodies can bind complement, and there can be a great variety of responses as a result of antibody-antigen interaction.

Finger: I cannot answer the parasitological part of your question. As I have mentioned, of course, in paramecium many reagents other than antibodies induce transformation—enzymes, temperature, shock, actinomycin D, puromycin, and so on.

With regard to the killing of a cell by antibody, we still do not know the mechanism of death in paramecia that are immobilized by antibodies. By the way, this occurs with decomplemented serum. It can occur very rapidly, within less than a minute, and we have made some preliminary pictures with the EM to see precisely what may be happening. So far we have no clue at all to why the antibody in combination with the antigen should result in death. In more dilute concentrations of antibody there is no death, and there can be transformation of type.

Borsos: I mentioned the phytohemagglutinins because they react at the surface of cells. The other substances are probably not acting on the surface. Actinomycin is not; heat is not. Any one of these changes induced is not due to surface action. However, antibodies do act on the surface.

Finger: As I have tried to stress, we think that the antibodies that are having their effect on transformation may really not be acting at the surface.

Bruce-Chwatt: I should like to put to Dr. Taliaferro a question that has puzzled parasitologists since 1948. What explanation can he give for the fact that the pre-erythrocytic or extra-erythrocytic schizont in the liver of mammalian parasites does not produce any cellular response? Dr. Brown mentioned that in PE forms the development is so fast that there may have been no time for an immunocellular response, but this is not so when it comes to secondary tissue stages, which produce no cellular response in the liver.

Moderater: Dr. Taliaferro, would you care to comment?

Taliaferro: I am afraid I can't.

Moderator: We shall continue now with the papers by Dr. Smithers and Dr. Remington. Again, the discussion will follow both presentations.

THE INDUCTION AND NATURE OF ANTIBODY RESPONSE TO PARASITES

S. R. Smithers

The literature on antibodies in parasitic infections is extensive, and nothing like a complete review of this subject is possible in the time available. I am therefore going to deal with the subject in broad outline, drawing examples from various infections that illustrate important concepts. Throughout, I shall concentrate on the significance of antibodies in resistance to infection.

Changes in the immunoglobulins in parasitic diseases

Raised immunoglobulin levels in parasitic diseases are common. Unfortunately, most of the studies on this subject were made before the recognition of the immunoglobulins as four distinct types—IgG, IgM, IgA, and IgD—and in most infections the relative increase in each immunoglobulin type is not known.

In experimental and in human schistosomiasis there are large increases (as much as twofold) in the γ and β globulin levels, as well as increases in the α_2 globulins (20, 21, 29, 61, 68, 79, 89). These increases occur at the time of egg deposition by the parasite and are probably due to the increase in antigenic stimulation that results from the excretions and secretions of the fully mature worms and the larval stages within the eggs. However, some alteration in the globulin values may be due to other causes. The α_2 globulin increase, for example, may be the result of tissue damage caused by the deposition of eggs in the liver and intestine (4), and the increase in the level of macroglobulin in chronic schistosomiasis that has been reported (6, 21) may be due to splenomegaly (44). In rhesus monkeys exposed to *Schistosoma mansoni*, the increase in immunoglobulin at the time of egg deposition occurs in the absence of an increase in macroglobulin. Figure 1 shows the average macroglobulin values (determined by ultracentrifugation) from three monkeys estimated on the day of exposure to 1,000 *S. mansoni* cercariae, four weeks later, and eight weeks later. Although the total immunoglobulin value had risen at eight weeks, the macroglobulin value had remained constant.

In trypanosomal and malarial infections, the IgG, IgA, and IgM levels are all raised (47, 77, 88). Especially high IgM levels are reached early in the infection, and it has been suggested

FIGURE 1. Average gamma globulin values and macroglobulin values of three rhesus monkeys after exposure to 1000 cercariae of *S. mansoni*.

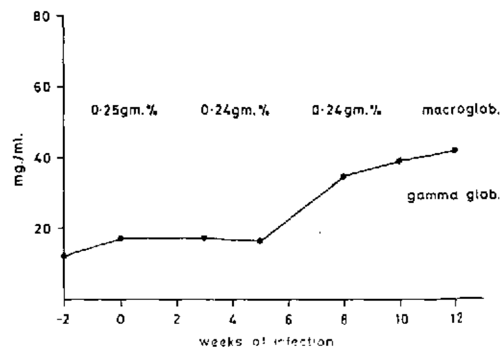
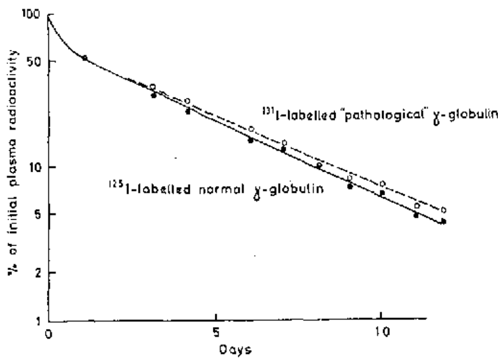


FIGURE 2. Catabolism of normal IgG and "pathological" IgG in a rhesus monkey five weeks after exposure to 1000 cercariae of *S. mansoni.*



that frequent antigenic variation of the malarial and trypanosomal parasite may contribute to the raised IgM levels (7). Each successive variant antigen is likely to stimulate the production of a new IgM antibody, giving rise to higher IgM levels.

The increase in IgG is probably not due to an increase in specific antibody. Curtain and his co-workers (14) found, by absorbing "malarial serum" through Sephadex conjugated with disintegrated malarial parasites, that the malarial antibody represented only 6 to 11 per cent of the total γ_2 7S antibody.

Somewhat similar results have been obtained in our own laboratory by a different method. We have taken IgG from a monkey infected with schistosomes—that is, IgG taken after the increase in concentration had occurred—and injected it together with normal IgG into a monkey that had been infected 5 weeks previously so that the parasites had just begun to mature and lay eggs and therefore to release large amounts of antigen. The "pathological" IgG and the normal IgG were labeled with different isotopes of iodine so that their rate of catabolism could be separately followed. If the "pathological" IgG had consisted mainly of antibodies specific to schistosome antigen, then it might have been expected to disappear more quickly from the circulation of the infected animal, because of its combination with antigen, than the normal globulin did. In fact, as can be seen from Figure 2, the two IgG's were catabolized at the same rate. Within the limits of our experimental technique we can say from this experiment that less than 5 per cent of the "pathological" IgG was specific to schistosome antigens. Similar experiments were carried out in malaria- and in trypanosome-infected monkeys with substantially similar results (Freeman, Smithers, Targett, and Walker; manuscript in preparation).

The nonfunctional nature and the nonspecificity of the hypergammaglobulinemia response are shown by the fact that resistance to schistosome infection in rhesus monkeys can develop in the absence of raised immunoglobulin levels. Monkeys exposed to worms of only one sex of schistosome, or to attentuated cercariae (infections in which the female worms do not mature and eggs are not produced), do not show any alteration in their serum proteins, but nevertheless resistance against a normal infection does develop (29, 76). In vaccination experiments against malaria in monkeys, not all the monkeys that showed an increase in gamma globulin as a result of vaccination were protected against the infection (86).

It does appear, therefore, that the large increases in immunoglobulin values as a result of parasitic infection may be due not to the production of specific antibody but to other indirect causes. Askonas and Humphrey (2), for example, found that the stimulation of tissues of hyperimmunized rabbits by antigen produced at least as much nonspecific immunoglobulin as antibody; and Humphrey (31) can see no reason why plasma cells should not be stimulated to secrete immunoglobulins that do not necessarily bear the imprint of any particular antigen.

Other reasons for hypergammaglobulinemia can be found; IgM levels are elevated in many cases of splenomegaly (44), and α_2 globulin levels may be increased when tissue damage occurs (4). It has been suggested that the raised levels of macroglobulin in the serum of patients with malaria, trypanosomiasis, and leishmaniasis may be due to a 19S antibody against denaturated IgG—a rheumatoid-factor-like globulin (27). Immunoconglutinins have

been demonstrated in *Trypanosoma brucei* infections of rabbits and cats (32) and may well contribute to raised immunoglobulin levels.

The etiology of hypergammaglobulinemia in parasitic infections is obviously complex, and yet it is fundamental to our knowledge of parasite immunity and pathology and of antibody production. With the recent expansion of our knowledge of the immunoglobulins and with techniques becoming available for their easy assay, we should be able to make considerable advances in our understanding of the significance of raised immunoglobulins in the pathology and immunity of parasitic diseases.

Specific antibody response to parasites

Protozoa

The liberation of large amounts of varied antigens from parasitic infections induces a complex antibody response. Almost every type of antibody known in classical immunology has been described. Thus, precipitins, lysins, agglutinins, opsonins, complement-fixing antibodies are all known in parasitological infections, and there are some antibodies peculiar to parasitology, such as ablastin in *T. lewisi* infections. Undoubtedly many antigens will induce an antibody that will agglutinate, precipitate fix complement, lyse, and so forth, depending on the appropriate conditions. Many of these *in vitro* reactions may therefore be manifestations of the same antibody under different conditions; on the other hand, one antigen may induce more than one antibody. It is easy to see, therefore, why the antibody response to parasites is complex and still poorly understood.

The induction of parasite antibody depends on the natural history of the infection. This is well illustrated in leishmaniasis, a disease caused by an intracellular protozoan. There are two forms of the disease: a cutaneous form resulting in a local infection of the skin, and a serious systemic infection, visceral leishmaniasis, involving the spleen and other reticulo-endothelial organs. In cutaneous leishmaniasis circulating antibody has not yet been demonstrated, although delayed skin reactions are obtained early in the infection. In visceral leishmaniasis, on the other hand, circulating antibodies are readily demonstrated even if there is little or no immunity to this disease (1). In malaria it is difficult to detect antibody before the parasites can be demonstrated in the red cells, but during the erythrocytic cycle antibody is easily detected (87). This does not imply that the other stages lack antigenicity, but rather that antigen is present in too low a concentration to induce a response (62). It is well known that when antigen is presented intravascularly, the spleen is of major importance in antibody production, and, as would be expected, splenectomy in malaria and African trypanosome infections results in an increase in the parasitemia. On the other hand, splenectomy has little effect in chronic Chagas' disease where the parasites are found within tissue cells (23).

At present it is difficult to correlate any one antibody with a protective role. In malaria it has been shown that neither the complement-fixation titer nor the fluorescent-antibody titer is necessarily correlated with protection (22, 86). It is known, however, from the successful passive transfer experiments of Coggeshall and Kumm (9) and Cohen and McGregor (10), that humoral agents must be playing an important role in protection. In the latter work the protective agent was in the IgG fraction of the immunoglobulin. Even so, large amounts of transferred IgG are necessary in order to demonstrate its protective effect. The reasons for the difficulty in demonstrating passive transfer are not clear. It has been suggested that the antibodies formed in the spleen, liver, and bone marrow occur in sufficient quantities to be active in those sites but are insufficient after dilution in the serum to be passively transferred (84). Apart from this possibility, passive transfer is complicated by the fact that a given species of *Plasmodium* may consist of a number of races or strains that may differ in their antigenicity. Cross-immunity may exist between strains of the same species, although to a lesser extent than against the homologous strain (83).

In trypanosomiasis, most of the detailed stud-

ies have been on the nonpathogenic rodent forms, against which three types of protective antibody have been described. One is ablastin, a 7S antibody that inhibits division of the trypanosomes without being immediately lethal; it can be absorbed from serum only with difficulty. The other two antibodies are trypanocidal; one is a 7S and the other a 19S immunoglobulin (15). In the African trypanosomes of human and veterinary importance, the antibody response is complicated by the antigenic variant strains that appear as a counter to antibody response (24). However, serum from animals with *T. gambiense* is able to confer passive immunity, but recent work has suggested that the agglutinating antibody present in such serum is not the antibody responsible for protection (71).

The antibody response in Chagas' disease is still poorly understood. In the acute form of the disease trypanosomes circulate in the blood stream, whereas in the chronic stages they are found only within cells. Lysins (16), precipitins (72), and fluorescent-antibody titers (93) have been demonstrated in sera from infected animals, but it is not known which, if any, of these antibodies has a protective function. Resistance has been conferred by transferring serum from infected hosts in mice and in rats (13, 41, 72). It is believed, however, that the transferred serum affects only the parasites circulating in the blood; the intracellular stage is probably unaffected by the immune serum. Pizzi believes that phagocytosis is an important defensive mechanism in the acute stage, and it is likely that the process is assisted by the action of an opsonin-like antibody; lysins apparently do not develop during the acute stage (60). Recently it was found that in a rhesus monkey infected with *T. cruzi*, the IgM level rose to a maximum on the tenth day of infection and then fell over a period of two weeks (unlike African trypanosome infections, in which the IgM level remains high throughout the disease) (93). This period roughly corresponded to the acute stage of the infection. The rhesus monkey or other primates may prove useful experimental animals for studying Chagas' disease.

It is clear that many antibodies are induced by protozoal infections, but most of these antibodies have no protective function. In most infections a humoral protective agent has been detected by passive transfer of serum, but the type of antibody responsible and the possible and likely interplay between cellular and humoral factors remain largely unknown. Little is known about the immune response to chronic Chagas' disease, in which the parasites are located in tissue cells, particularly heart muscle. It is conceivable that the immune reaction at this stage will enhance rather than reduce the pathology of the disease (60). According to Mackaness (45), organisms that can survive and multiply within host macrophages caused delayed-type hypersensitivity toward the corresponding microbial antigens. *Leishmania*, and to a lesser extent *T. cruzi* (17), are found within host macrophages, and it may be that investigations into cell-bound immunity would yield significant results in these infections.

Helminths

Much of what has been said about protozoal infections applies to helminthiasis. A wide variety of antigens is liberated from worm parasites, and a complex antibody response consisting of precipitating, agglutinating, complement-fixing, immobilizing, skin-sensitizing, nonprecipitating, and blocking antibodies is produced. In helminthiasis, unlike protozoal infections, lysins have not been detected. The lysis of a relatively large organism with a strong impermeable cuticle, such as a nematode, is probably outside the bounds of antibody capability. Schistosomes, however, have no cuticle as such but a living cytoplasmic membrane (42) that is probably concerned in the uptake of nutriment from the host (73). It is possible that antibody and complement could damage the schistosome surface, although no lysis of larval or adult schistosomes by specific antibody has been observed.

In schistosome infections various antibodies are produced (58, 81). Peak antibody titers

generally appear when the parasite matures and eggs are deposited in the tissues (50, 67, 75); it is at this stage that there is a sudden increase in the liberation of antigen. If, however, sensitive techniques such as complement fixation or fluorescent-labeled antibody are used, other antibodies can be demonstrated earlier in infection (30, 36, 46).

Antibody reactions have been described around living schistosomes *in vitro;* the cercarienhüllen reaction or CHR antibody is manifested as an envelope-like structure around cercariae when placed in specific antiserum (91), and circumoval precipitins or the COP reaction is seen when schistosome eggs are incubated in schistosome antiserum (57, 63). There is one report of precipitates forming around the oral sucker of three-week-old schistosomula when cultured in immune baboon serum (64). Quite recently it has been shown that the Hoeppli phenomenon, which is sometimes seen around schistosome eggs in host tissue, is an antigen-antibody complex (43).

Apart from the usual display of antibodies normally present in helminth infections, precipitates have been clearly demonstrated around the oral and excretory orifices when nematodes are incubated in immune serum (18, 48, 56, 59, 65, 69, 74). Jackson, using fluorescent-labeled antibody, demonstrated that these precipitates are the result of antigen-antibody combination in infections of *Nippostrongylus braziliensis* and *Trichinella spiralis* (34, 35). Taliaferro and Sarles have also shown precipitates in and around *N. braziliensis* adults and larvae in infected rats (85).

What significance have anti-worm antibodies in protecting the host against the parasite? At the present time no demonstrable antibody in helminth infections can be directly correlated with acquired resistance. In schistosome infections the CHR, COP, complement-fixing antibody, fluorescent-antibody titers, skin tests, and various precipitins show no relation to protection (33, 76, 92). Animals usually produce a marked antibody response after the injection of dead parasite material, but protection against reinfection cannot be induced in this way. Moreover, resistance can be induced in the absence of eggs and fully mature worms (that is, in the absence of the stages of the life cycle that induce the greatest antibody response) as easily as in a normal infection (76, 78).

Ogilvie has shown that the precipitates that develop around *N. braziliensis* larvae when incubated in immune rat serum are no guide to immunity. These precipitates are not detected after one and sometimes two normal infections, although the rats are resistant to challenge at this time. On the other hand, they do develop in the serum from rats infected with extracts of adult worms in adjuvant, yet rats treated in this way are not resistant to challenge (52).

Thus, as in protozoal infections, a host of antibodies is produced but their role in protection is not clear. In protozoal diseases at least, a protective role by humoral factors can be demonstrated, with some difficulty, by the transfer of immune serum to a normal animal. In schistosomiasis, although many attempts have been made to transfer protection with serum from resistant animals, most have failed (80); and, as Kagan has pointed out, even in the few successful passive-transfer experiments (where only slight protection was conferred) absorption experiments have not been performed to determine that specific antibodies were responsible for the effect (40).

In several nematode infections passive transfer of protection with serum has been demonstrated (8, 12, 25, 37, 65, 70, 82, 90). But protection obtained in this way is seldom comparable to that produced by an active infection. Even when enormous quantities of serum are given, the infection is at best only damped down, the result being a slight reduction in worm take or egg output. Painstaking work by my colleagues has highlighted some of the difficulties of demonstrating passive transfer; during the past three years, 18 pools of immune serum from rats immunized against *N. braziliensis* by similar infection schedules have been tested for their protective properties. Only 11 of the serum pools have given passive protection, and the efficacy of each positive pool showed considerable variation (Ogilvie and

Jones, personal communication). Similar experiences have been reported by Crandall working with Ascaris in mice (11) and by Miller working with hookworm in dogs (49).

These results lend support to Stirewalt's conclusion that lack of passive transfer in schistosomiasis indicates not so much an absence or ineffectiveness of the antibodies as a lack of understanding on our part of how they function and how to handle them (80). In this connection, one is reminded of the recent reports of cytophilic antibodies, a term applied to globular components of antiserum that become attached to certain cells in such a way that the cells are subsequently capable of specifically absorbing antigen (51). Transfer of guinea pig cytophilic antibody is rapidly cleared from the circulation of recipient guinea pigs; only a small fraction of the injected cytophilic antibody is detectable after 24 hours, whereas the more conventional antibodies are still present in relatively high titers at this time (38). There is also evidence that conventional antibodies may even suppress phenomena in which cell-bound antibodies play a part, and Nelson and Boyden suggest that passive transfer with serum might be possible if the serum contained an unusually large amount of cytophilic antibody and an unusually small amount of conventional antibody (51). No cytophilic antibodies have as yet been described in parasitic infections, but a search for them could prove profitable.

Reagins. An interesting feature of the antibody response to helminth infections has been the subject of recent research. It has long been known that in helminth infections of man, an immediate-type skin reaction is elicited by intradermal injection of homologous antigen. Skin tests have been widely used in diagnostic and survey work, but it was perhaps not generally appreciated by parasitologists that such immediate reactions are mediated by a special class of antibodies called reagins.

Recently reagin-like antibodies have been demonstrated in experimental infections of helminths; in rats infected with *Nippostrongylus* (53, 54); in rats and monkeys infected with *Schistosoma* (19, 28, 55, 66); in rabbits infected with *Schistosoma* (5) and with several species of *Ascaroids* (26); in rats infected with the filarial parasite *Litomosoides carinii* (Worms, personal communication); and in sheep infected with *Trichostrongylus* (Ogilvie, personal communication). Reagin-like antibodies of rats, rabbits and monkeys are similar in properties to the reagins of man (5, 19).

Reagins are heat-labile, nonprecipitating antibodies that possess the property of prolonged tissue fixation to homologous skin or to the skin of closely related species. They appear to be slightly larger in molecular size than 7S gamma globulins and have fast electrophoretic mobility (19, 39).

The induction and nature of reagins in experimental helminth infections has been investigated mainly in rats infected with *Nippostrongylus* and in monkeys infected with *Schistosoma.* It is difficult to induce reagin formation by the inoculation of dead parasite material, although reagins are readily induced by a viable infection. In rats infected with *Nippostrongylus,* reagins appear in the serum about the time the animals acquire resistance and eliminate the majority of their worms. Further infections stimulate an anamnestic rise in reagin production, but if reagins are stimulated by vaccination with worm extracts a second vaccination will not induce an anamnestic response (54). In rhesus monkeys infected with S. *mansoni,* reagins appear any time after the sixth week of the first infection, but their appearance at this stage is transitory, for they disappear from the serum within the next two to four weeks. Subsequent challenge with cercariae, however, causes their reappearance within two weeks; thereafter they may persist for long periods (19).

The evidence so far is against the involvement of reagins in any protective function in monkeys infected with *Schistosoma* (19, 28). In rats infected with *Nippostrongylus,* the evidence at the moment suggests that reagins are not protective in the usual sense—that is, in initiating direct damage to the worm—but may be involved in the sudden termination of prim-

ary worm infections by an anaphylactic mechanism (3, 39).

The stimulation of reaginic antibodies by helminth infections poses some fascinating problems. Why are reagins produced in response to helminths as opposed to other parasites? Why are reagins induced only by the living infection process and not by the injection of helminth antigen? What is the link between helminthic infections and allergic diseases such as asthma and hay fever? Have reagins a significant role in the host-parasite relationship, or is their appearance just another misdirected function of the immunological mechanism? Answers to these questions would be of interest to allergists as well as to parasitologists.

Comment

I have pointed out in this brief review that it has seldom been possible to correlate antibody response with protective immunity in parasitic infections. In no host-parasite system (except *T. lewisi* in the rat) has protective antibody been characterized in terms of its biological function.

It follows that parasitologists are still faced with two basic problems in spite of the large amount of work done in this field: first, for the great majority of parasitic infections it is not yet possible to induce resistance by a safe and effective vaccine; second, although acquired immunity is common in parasitic infections, we know almost nothing of how it acts.

The attack on these problems is conducted along two main lines, which derive from classical immunology; these are active immunization by antigens and passive immunization by antiserum. Both approaches are aimed ultimately at the indentification of the functional antigens. Thus living parasites actively immunize their hosts, but living parasites are complex systems and must be simplified by fractionation and isolation of parasite antigens, with the protective power of the antigens determined at each step. Ultimately, pure antigens will be obtained that not only will vaccinate the host against the parasite but also may be characterized in terms of parasite physiology. As regards passive immunity, where this is possible, the protective serum must be fractionated in terms of the specific immunoglobulins. Purified functional antibody can then be absorbed with parasite antigens and in this way the functional antigens may also be identified.

These approaches are obvious to immunologists, who may well wonder why parasitologists have not already solved their problems. We can only plead that in practice the difficulties are enormous. To begin with, it is usually impossible to induce a high-grade protective immunity with nonliving parasite material. In the protozoa, this may be due to antigenic variation of the parasite; in helminths, it is probably due to the small amount of functional antigen present in dead worms. In any case, it is a hard task to attempt to isolate functional antigens when the starting material will not itself stimulate protective immunity. Again, in many cases it is very difficult to induce passive protection with serum; in some host-parasite systems it seems impossible, and in others the protection is erratic and requires very large amounts of serum. Thus the isolation of functional antibody is severely hampered.

It is small wonder, then, that many parasitologists, including myself, tend to skate around these two basic problems. We must, however, be continually aware of these problems and our own inadequacy in solving them. We must face up to the fact that it is little use analyzing the antibody response without attempting to relate it to the biology of the infection, and it is little use analyzing parasitic antigens without attempting to relate them to the stimulation of active immunity.

CHARACTERIZATION OF ANTIBODIES TO PARASITES*

Jack S. Remington

In marked contrast to the voluminous literature on the immunochemical and physicochemical characterization of antibodies formed in response to infection with a variety of bacteria and viruses, and to numerous pure antigens, relatively little is known about the characteristics of antibodies formed in response to infection with protozoa and helminths. Multiple serologic tests are available for the diagnosis of parasitic diseases, but we lack knowledge of the nature of the antibodies that participate in these reactions. Such characterization would not necessarily be of academic interest only but might shed additional light on the mechanisms of parasitism and host response.

When I saw the proposed title of my talk, I realized that of necessity the information discussed would be largely prospective in nature rather than both prospective and retrospective, as is possible with the subjects covered by the other discussants. In many ways this is an advantage—in an area as embryonic as this it is often useful to direct one's attention to what might be accomplished in the future.

The characterization of antibodies is a vast area of research and includes the definition of primary, secondary, tertiary, and quaternary structure; molecular and genetic variations; the determination of protein-bound carbohydrates, synthesis, distribution and turnover, serologic reactivity and specificity, and so forth. Here I shall concern myself solely with methods for immunochemical and/or physicochemical characterization and thus with recognition of the three major immunoglobulin classes of antibody —IgG, IgA, and IgM. Suitable references are given for each method.

First I should like to mention the data available on the characterization of antibodies in three parasitic infections. In 1965 Abele *et al.* (1) published their findings on alterations in serum proteins and 19S antibody production during the course of induced malarial infections in man. These authors noted a consistent increase in IgM in immunoelectrophoretic patterns of serum obtained from volunteers infected with *Plasmodium vivax* and *Plasmodium cynomolgi.* Sera were separated by gel filtration on columns of Sephadex G-200, and the fractions were tested for antibody activity by means of the fluorescent-antibody test using a fluorescein-labeled rabbit anti-human immunoglobulin antiserum. In addition, reductive cleavage with mercaptoethanol was used to help differentiate IgG and IgM antibodies. In several of the volunteers antibody activity was confined to the IgM fractions of serum during the first few days of antibody production, but thereafter was found in both IgM and IgG. Approximately 40 days after infection antibody activity was present only in IgG. Of significance for our discussion here is the use by the authors of both blood-induced and sporozoite-induced infections and the variability in response in the different volunteers. For example, in one case of sporozoite-induced infection antibody activity was demonstrable only in IgG and never in IgM frac-

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tions. Characterization of the antibodies in these cases has revealed previously unrecognized parameters requiring further exploration, such as the relative efficacy of these different classes of antibodies in protection against infections (2), their reactivity and specificity in different serologic tests, the antigenic structures stimulating their production, and the relation of inoculum size to class of immunoglobulin response.

Recently we have demonstrated the presence of IgM toxoplasma antibodies in humans with acute acquired and congenital toxoplasmosis (3). A complete review of our data has recently been submitted for publication and will not be described here (4). Characterization of toxoplasma antibodies was performed to determine whether a demonstration of IgM toxoplasma antibodies would prove diagnostically significant. Since IgG toxoplasma antibodies freely traverse the placenta, their presence in the newborn may only reflect maternal infection (5). IgM antibodies do not normally traverse the placenta but may be formed *in utero* in response to infection (6). Their demonstration in a newborn, in the absence of a placental leak, has proved diagnostic of congenital toxoplasmosis.

Initially, the demonstration of IgM toxoplasma antibodies was accomplished by the use of sucrose density gradient ultracentrifugation and reductive cleavage. Recently we developed a modification of the indirect fluorescent-antibody technique, using a fluorescein-tagged antiserum specific for IgM. This technique can be employed in any laboratory that has a fluorescent microscope and can be completed within about two hours. It may prove useful in studies of characterization of antibodies to other parasites and in the diagnosis of other infections —especially in the newborn. A description appears on pages 55 and 56.

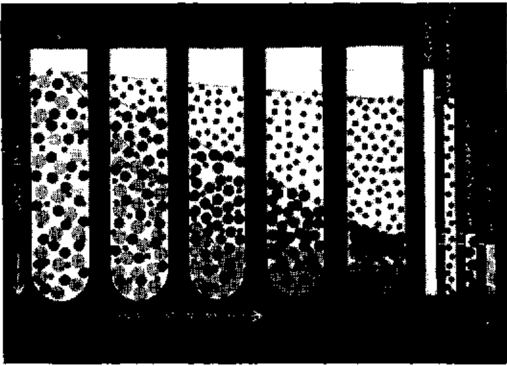
We are now employing the technique in a prospective study of 5,000 newborns to determine the incidence of congenital toxoplasmosis in the San Francisco Bay Area. Because of the wide clinical spectrum of congenital toxoplasmosis in the newborn (7) and the presence of maternally transmitted antibodies, such a study could not be performed previously.

Crandall, Cebra and Crandall have recently described the relative proportion of IgG-, IgA-, and IgM-containing cells in rabbit tissues during experimental trichinosis (8). Direct counting of cells containing two different immunoglobulin classes in a single tissue section was accomplished by staining with pairs of immunofluorescent reagents, specifically reactive with γ-, μ-, or α-immunoglobulin heavy chains and labeled with contrasting fluorochromes.

By the use of two different pairs of reagents on adjacent sections, the relative numbers of cells containing IgG, IgM and IgA were calculated. The most striking change in the relative proportions of immunoglobulin-containing cells in the intestinal mucosa of infected animals was an increase in cells containing IgM in early infections. Later in the infection, cells containing IgG made up a larger proportion of the total IgM plus IgG population. This change in relation to time after infection correlates with the sequence of appearance of the immunoglobulin classes of humoral antibody after antigenic stimulation. The number of immunoglobulin-containing cells in the diaphragm suggested that skeletal muscle is an important site for immunoglobulin production in *Trichinella* infections. The authors detected humoral IgG and IgM antibodies to *Trichinella* but not IgA. Although this study was oriented mainly toward the study of cellular production of the various immunoglobulin classes of antibody, it can readily be seen that such an experimental approach to characterizing antibodies and antibody formation might be used in the study of host response to a variety of parasites.

There are large numbers of techniques now available for characterizing antibodies. No attempt will be made here to review or even mention all of them. Information about many of the techniques may be found in references (5), (9), and (10).

The characterization of antibodies in the three parasitic infections mentioned above illustrates what can be done in this area in the field of parasitology. Each of the techniques used in those studies had been previously employed to characterize antibodies formed



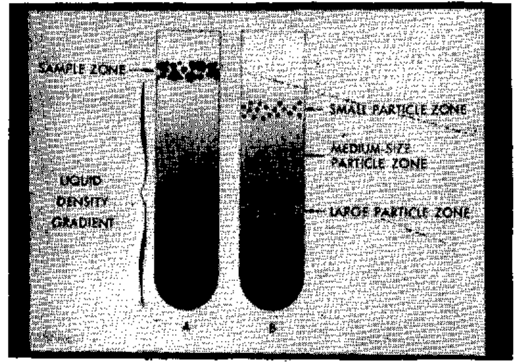


FIGURE 1A. Diagrammatic presentation of the sedimentation of particles in a swinging-bucket centrifuge tube. The distribution of particles initially is shown in tube A. Tubes B to E show the sedimentation of particles during centrifugation and illustrate the sources of cross contamination. The sedimentation rates of particle boundaries are indicated by the angled lines. The bars at the right indicate the distribution of solvent and particles in the last tube. FIGURE 1B. Rate-zonal centrifugation in a conventional swinging-bucket certrifuge tube. Initially a thin sample zone is layered over a liquid density gradient (A) with the tube at rest. After centrifugation (B) particles having different sedimentation rates are separated into zones at different density levels in the gradient. (Reproduced by permission from "Zonal Ultracentrifugation" by Norman G. Anderson, *Fractions*, No. 1, 1965, published by Spinco Division of Beckman Instruments, Inc.)

against many unrelated organisms and antigens. One of them, density gradient ultracentrifugation, is shown diagramatically in Figures 1A and 1B. For separating the various molecular species of immunoglobulins, gradients of sucrose are usually employed. In Figure 2 are shown

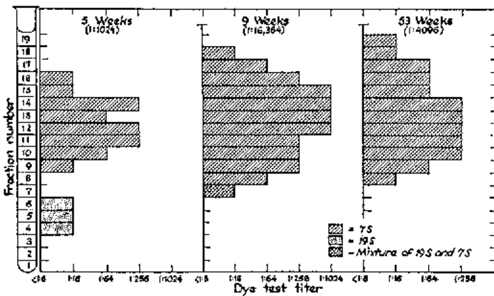


FIGURE 2. Separation of 19S and 7S dye test antibodies by sucrose density gradient ultracentrifugation in a case of acute toxoplasmosis. Ordinate = sucrose gradient fractions. 1 through 6 = IgM. 7 through 19 = IgG.

the results obtained by this method in a serum of a patient with acute acquired toxoplasmosis. Only IgM or 19S antibodies were demonstrable by this method at 5 weeks of illness, and only IgG or 7S antibodies were demonstrable at 9 and 53 weeks. This technique requires the use of a preparative ultracentrifuge, which is expensive and is not readily available in most laboratories now involved in research in parasitology. Another method for separating the immunoglobulins is gel filtration on columns of Sephadex (11, 12). An example of results obtained using this method is shown in Figure 3. Using this technique we not only were able to show the absence of cross-reactivity between heterophile and dye test antibodies but also learned something of the molecular size of these two antibodies in the serum samples employed (13). Although IgA antibodies were not looked for in this experiment, they usually reside in eluates collected between the first and second peaks.

To actually define an antibody as IgG, IgA, or IgM one must usually resort to studies employing antisera specific for the heavy chains of these three immunoglobulins. (There is a fourth class, IgD, but antibody activity has not yet been reported in it.) This area of immunology has already been reviewed very adequately and will not be discussed here (5, 9, 10, 14). The techniques are simple, and usually immunodiffusion in agar is employed. The antisera are available from a number of supply houses in the United States and Europe. Great care must be exercised to verify the stated specificity of any purchased antiserum. For this purpose controls (specific immunoglobulins) may be purchased or obtained by chromatographic pro-

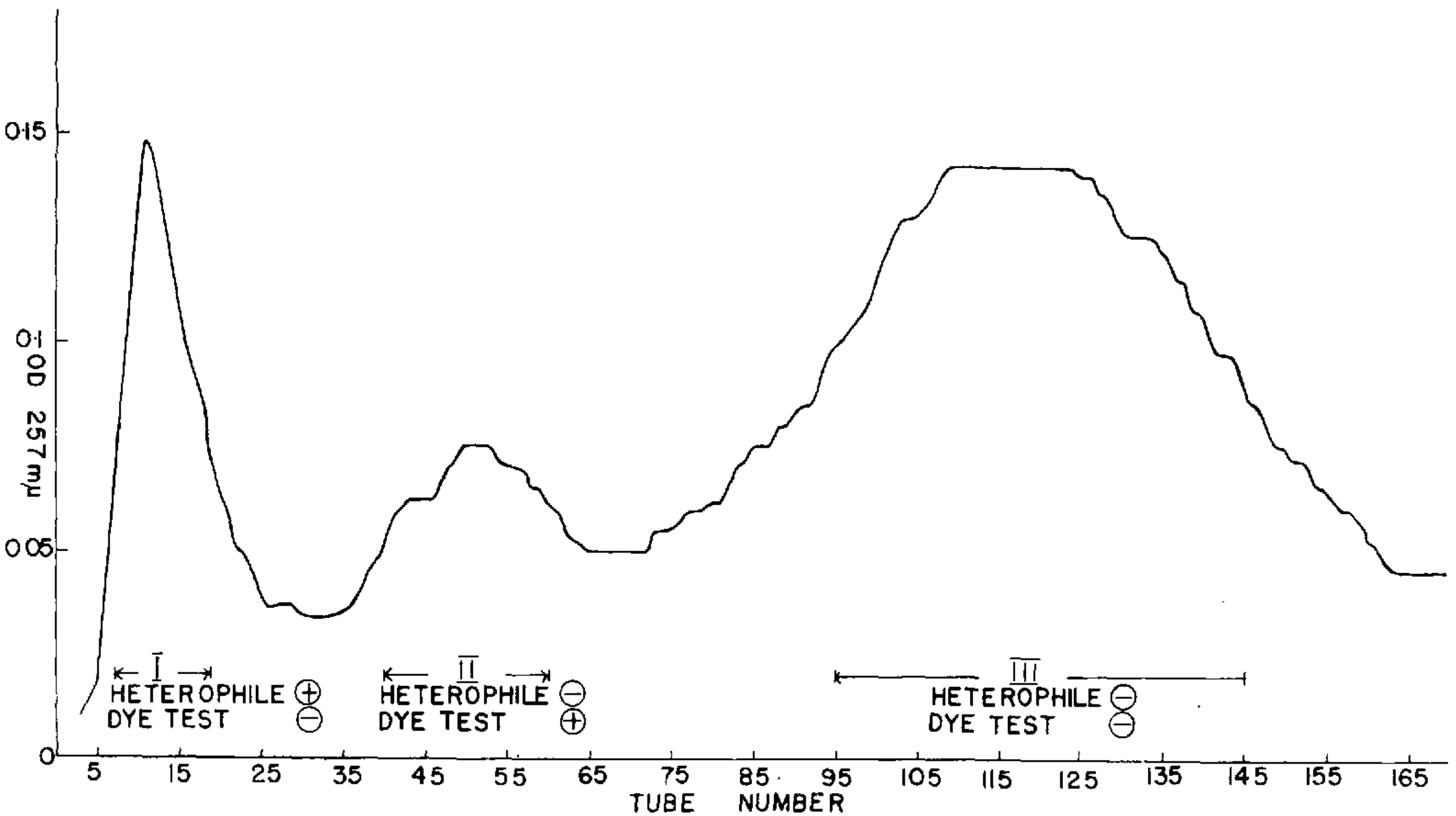


FIGURE 3. Elution pattern from Sephadex G-200 of a pool of sera containing heterophile and dye test antibodies. Eluting solvent: 0.15 M NaCl.

cedures. A number of investigators in the field are often willing to supply small amounts of these immunoglobulins and/or specific antisera.

It is a relatively simple matter to obtain serum IgG and IgM by chromatography on columns of diethylaminoethyl cellulose (DEAE). We first separate serum by starch block electrophoresis, an easy and inexpensive technique (10, 15). The γ-portion is then applied to a column of DEAE previously equilibrated with 0.0175 M phosphate buffer pH 6.3. The fraction eluted with that buffer contains only IgG. Then elution with 0.1 M phosphate buffer pH 5.8 results in a fraction containing IgA contaminated with IgG and IgM. Finally, elution with 0.4 M phosphate buffer in 2 M NaCl pH 5.2 produces a very purified IgM. Recycling and use of DEAE Sephadex followed by Sephadex G-200 will produce a purified IgA preparation (16). These immunoglobulins may then be used to immunize animals for obtaining specific antisera for controls in immunodiffusion studies, for adsorption of nonspecific antisera, and for inhibition and fluorescent-antibody studies.

A widely used and simple method for characterizing IgM antibodies is reductive cleavage of disulfide bonds with mercaptoethanol, which destroys IgM antibody activity (17, 18). In most instances such treatment of IgG antibodies results in no loss of antibody activity. We have used this method to differentiate between IgC and IgM dye and hemagglutination test antibodies for toxoplasma. The sera were incubated at 37°C for one hour in 0.02 M 2-mercaptoethanol. The samples and controls were then diluted immediately and run in the serologic tests. Alkylation with iodoacetamide is not necessary if the tests are run soon after treatment with 2-mercaptoethanol. Figure 4 shows the results of such treatment in the hemagglutination test in a serum from a case of acute acquired toxoplasmosis (4).

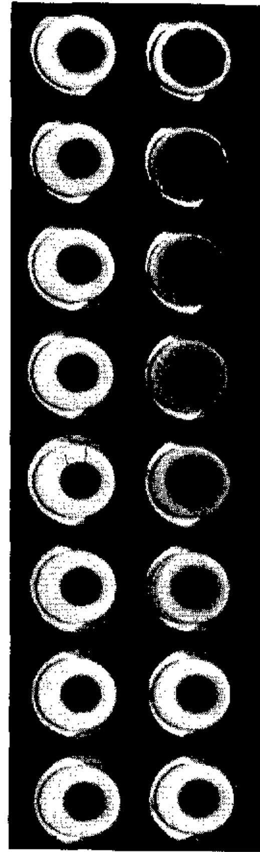


FIGURE 4. Hemagglutination pattern in serum of case of acute acquired toxoplasmosis. Right: No treatment of serum, titer = 1:400. Left: Serum treated with 2-mercaptoethanol, titer = <1:50.

Laboratories without a preparative or analytical ultracentrifuge can use another method —gel filtration (Sephadex) (19)—for determining molecular weights of antibodies or their fragments obtained by enzymatic degradation. There is an excellent linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume to the void volume of the columns. Using various forms of Sephadex, this relationship holds true for molecular weights from 13,000 to 225,000, and probably for considerably beyond.

The availability of antisera for the different classes of human immunoglobulins allows for the use of the indirect fluorescent-antibody technique to demonstrate IgG, IgA, or IgM antibodies, as was mentioned above for toxoplasma. Such a technique might be applied to trypanosomiasis, malaria, and schistosomiasis

and to many other parasitic diseases of man as well. In animal studies the problem is more involved, since specific antisera for the various classes of animal immunoglobulins are not readily available. Here it would be necessary to resort to preparing the purified immunoglobulins and their respective antisera.

These techniques lend themselves to the study not only of serum antibody and cellular production of immunoglobulins but also of antibodies of body secretions and excretions (15, 20, 21, 22). Relatively little is known of the characteristics of coproantibody response to intestinal parasites. About 90 per cent of the immunoglobulin-producing cells of the lamina propria of the duodenum and jejunum produce IgA (23). What is the role of this immunoglobulin in parasitic infections of the intestine? What is the sequence of the formation of antibody in the intestine to various parasites? Since little is known of the importance and function of IgA as exocrine antibody, studies employing amoebae and certain helminths may help clarify this subject.

Although it is not within the scope of this manuscript to cover extensively the techniques that might be used in such studies, sufficient references are given to allow one to become acquainted with them. It is unfortunate that these newer techniques, many of them relatively simple to perform, have not been used by more laboratories in the study of parasites and of parasitism. For years the immunology of parasitic diseases has been associated with histopathology and the study of antigens for diagnosis and immunization. In recent years tremendous advances have been made in the technology of imunochemistry—and thereby in our knowledge of immunity. In this country laboratories such as those of Drs. Elvio Sadun and Irving Kagan have served as an impetus to other workers in parasitology not previously oriented to immunology. With laboratory methods now at hand for the characterization of antibodies to parasites, it is hoped that such studies will be performed at least in part by, or in laboratories of, those with the greatest knowledge of the field of parasitology and appreciation of its problems—parasitologists.

Description of indirect fluorescent-antibody test used for rapid demonstration of IgM-toxoplasma antibodies

The technique employed is essentially that described by Walton, Benchoff, and Brooks (24). The RH strain of toxoplasma is obtained from the peritoneal fluids of three-day infected mice. The fluid is added to a small amount of phosphate-buffered NaCl pH 7.2 (PBS) containing 1 per cent formaldehyde and heparin. The suspension is mixed thoroughly with a 10-ml syringe and a No. 22 needle to disrupt cells containing organisms. The volume is then brought to 40 ml by adding additional PBS containing the formaldehyde and is centrifuged at 1,500 rpm for 10 minutes. The supernatant is discarded, and the sediment is resuspended and washed twice in the PBS-formaldehyde solution. After the second wash the sediment is resuspended in an amount of PBS-formaldehyde solution sufficient to give 100-200 organisms per high-power field when delivered to the 1 cm circled areas on the slide (24). If many leukocytes are present in the peritoneal fluid, the organisms may be separated by filtration through a Baird-Tatlock filter as described by Fulton and Spooner (25). However, by careful timing of the period between the inoculation of animals and the obtaining of peritoneal fluid, exudate may be obtained that contains relatively few leukocytes. Slides for future use may be frozen at —20° C for at least four months (the longest period tested).

PBS is used for diluting samples of serum and as a wash solution for the slides after each step in the procedure. The fluorescein-conjugated goat anti-human IgM antiserum was purchased from Hoechst Pharmaceuticals, Inc., Cincinnati, Ohio. Through the excellent cooperation of Mr. H. Jelinek of the Diagnostic Reagents Division, a number of such sera were tested for the presence of toxoplasma antibodies prior to conjugation with the fluorochrome. Lot F132 was found to be negative

(<1:2) in the dye test and was employed in all the studies we have performed to date. No precipitin arc resulted when this antiserum was reacted by double diffusion in agar gel against IgG. Reactions of this same antiserum with normal human serum resulted in a single precipitin arc that gave a reaction of identity with that formed by the reaction of the antiserum with IgM. Dilutions of the conjugated antiserum were made with a variation of the diluent recommended by Goldman (26), using PBS containing 1.2 per cent bovine serum albumin. The appropriate dilution of the antiserum was 1:100 to 1:150. The slides were examined with a Zeiss Fluorescence Photomicroscope using an Achromatic-aplanatic condenser with a BG 12 exciter filter and barrier filters 53 and 44. 12.5X oculars were used with the 40X objective and 1.25 optivar giving a magnification of 625X. The photographs were taken with high-speed Ektachrome daylight color film using the dark field condenser and BG 12 exciter filter and 65/50/41 barrier filters. The exposures were for 30 minutes with the 40X objective and 1.6 optivar giving a magnification of 800X.

The initial dilution of the serum samples was 1:8 or 1:10, and thereafter serial twofold dilutions were made. The titer of a given serum is reported as that dilution in which at least 50 per cent of the organisms in multiple fields appeared to have completely outlined cell walls. Two other types of fluorescence that were noted were the staining of one tip of the organism or fragmentary staining of the cell wall. Such reactions were not considered positive. Three controls were run each time the test was performed: (a) a serum negative (<1:8) in the dye test, (b) serial dilutions of a serum known to contain IgM dye test antibodies, and (c) a 1:8 dilution of a serum known to contain only IgC dye test antibodies (titer=1:256). In the IgM-fluorescent-antibody test controls (a) and (c) there was either no fluorescence of the organisms or a staining of some tips. Control (b) resulted in brightly fluorescent rims at a titer of 1:8 through 1:128, and at a titer of 1:256 there were rare faint rims or no fluorescence. As a control in some experiments a fluorescein-conjugated goat anti-human IgG antiserum (Lot 7V-818) obtained from Hyland Laboratories, Los Angeles, California, was used in a dilution of 1:100.

To test the specificity of the fluorescein-tagged anti-IgM antiserum, 34 sera with IgM-toxoplasma antibodies were treated with 2-mercaptoethanol (2-ME) and thereafter tested in the IgM-fluorescent-antibody test. The dye test titers in these sera ranged from 1:1024 to 1:65000. Treatment with 2-ME completely destroyed the ability of sera with IgM-fluorescent-antibody test titers to cause fluorescence. In contrast, this treatment did not affect their ability to cause brilliant fluorescence when a fluorescein-tagged anti-IgG antiserum was employed. Pooled IgG isolated from sera of human cases containing both IgG and IgM-toxoplasma antibodies was tested in the IgM-fluorescent-antibody test. The titer of the pooled IgG in the dye test was 1:32000. This IgG gave no fluorescence in the IgM-fluorescent-antibody test.

Moderator: Before introducing the discussant, I should like to call attention to one point that has not been mentioned by the speakers: the possible role of antienzymes in parasitic immunity. I think this possibility was raised fifteen years ago by Chandler when he suggested that the stunting of worms in the immune host was the effect of the antibody on the metabolism of the parasite. The idea was further developed by a group of workers at the University of Chicago, who showed that while many serological reactions observed with *Schistosoma mansoni*—such as complement fixation, circumoval precipitation, and miracidial and cercarial agglutination—are definitely associated with IgG antibodies, the inhibition of collagenase activity in cercariae depends on a heat-stable factor migrating as an alpha globulin. The comparative susceptibility of different species ran parallel to the level of the inhibitor, which would affect the penetration and development of cercariae.

It is quite possible, however, that antibodies to enzymes play an important role in the

penetration of larvae or affect their life cycle inside the immune host. It is known, for example, that the sera of infected rats can neutralize lipase of *Nippostrongylus* larvae and that the sera of infected dogs neutralize the proteolytic activity of esophageal extracts of adults and larvae of *Ancylostoma caninum.*

Leucin aminopepsidase is produced under the molting stimuli in both *Haemonchus contortus* and *Trichostrongylus colubriformus* and seems to be responsible for the attack of the larval sheath leading to its liberation.

There are people working on enzymes of *Schistosoma mansoni,* and at least two groups are working with the techniques developed by Uriel in France using electrophoresis or immunoelectrophoresis on agarose gel. I refer to the work recently published in France by a group of investigators in Lille, who report the presence in the adult *Schistosoma mansoni* of four dehydrogenases (mallic, lactic, alanine, and glucose-6-phosphate dehydrogenases), alkaline phosphatase, chymotrypsin, and carbonic anhydrase. These same enzymes were found in *Fasciola hepatica.*

In our laboratory we have been interested particularly in cercariae, and our experiments show positive results for leucin aminopeptidase, carboxypeptidase A, chymotrypsin, and alkaline protease.

As a complement to what has been said by the speakers on the antibodies to parasites, I thought, something should be added about the antienzymes.

Now I call on Dr. Goodman to start the discussion and Dr. Nussenzweig to follow.

DISCUSSION

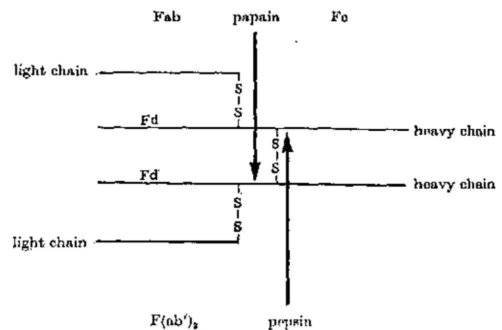
Howard C. Goodman

It is interesting to contrast the situation in 1937, when Drs. Kabat and Tiselius showed that antibodies were in the gamma globulin fraction, with the present evidence of great heterogeneity of the globulins with antibody activity that we call immunoglobulins. There are not only the three major classes—IgG, IgM, and IgA—that Dr. Remington described, but also a fourth class, IgD, and a possible fifth class, IgE.

The rapid advances in our knowledge about the *structure* of immunoglobulins and of the structural basis for the heterogeneity of immunoglobulins, documented in WHO nomenclature publications (10, 11, 12), have far outstripped advances in our understanding of the *functional* significance of the rather overwhelming heterogeneity of classes and subclasses of immunoglobulins.

The heterogeneity within even a single major class of immunoglobulins, the IgG, is rather overwhelming (Figure 1). There are not only the two different types, κ and λ, of light chains (and at least the four different kinds of heavy chains which account for the four subgroups of IgG recognizable at present), but also the different allotypic specificities. Evidence exists for what has been termed idiotypic specificity; the evidence suggests that there are structural components peculiar to the antibodies made to one given antigen by one individual or group of individuals. The heterogeneity is also expressed by differences in electrophoretic mobility and in solubility (euglobulins and pseudoglobulins) of molecules within the IgG class. Finally, there is the division into the constant and variable

FIGURE 1. Diagrammatic four-chain structure of the immunoglobulin molecule showing the probable sites of cleavage by papain and pepsin. The number of inter-heavy chain disulphide bridges has not been established with certainty. (From S. Cohen, General Structure and Heterogeneity of Immunoglobulins, *Proc. R. Soc.* [Ser. B], 166; p. 114.)



(n-terminal) portion of both the light and the heavy chains. It is the variable portion of the Fd part of the heavy chain and the variable portion of the light chains that are thought to be related to the one biological property common to *all* antibodies: that of combining with specific antigens. If, in accord with present ideas, the specificity of the antigen-combining site of the antibody produced by tertiary folding of the polypeptide chains is determined by primary structure, then parts of the variable portion of the chains must have different amino acid sequences for each and every antibody specificity.

Thus we are faced with a staggering heterogeneity of amino acid sequences that present a real challenge to present theories about the genetic control of the synthesis of polypeptide chains. In fact, the challenge has become so intriguing that the molecular biologists have

decided to devote this year's Cold Spring Harbor Symposium in June to the consideration of the genetic basis for the synthesis of immunoglobulin molecules.

Returning to the classes of immunoglobulins in human sera, it seems established that the general basis for differences in the four major classes of immunoglobulins depends upon the four different kinds of heavy chains characteristic for each class, the γ, α, μ and δ. There are structural variations within each class of heavy chains, accounting for the currently recognized subclasses of IgG, IgA, and IgM (which subclasses obviously do not mark the end of our ability to subdivide major classes of immunoglobulins). The existence of such subclasses of immunoglobulins may provide an explanation for some of the conflicting results on the biological activities of antibodies that were generally considered to be within one class, IgG, which can really be regarded as including a whole family of immunoglobulins.

One of the major challenges in immunology is to correlate the new knowledge about differences in amino acid sequence resulting in the different polypeptide chains characteristic of different kinds of immunoglobulins with the different biological functions that presumably are also characteristic of the different kinds of immunoglobulins. We are at the beginning of what might be termed "molecular immunology," of being able to understand the molecular basis for what hitherto have necessarily often been descriptive phenomena of immunology, such as complement fixation, tissue sensitization and anaphylaxis, cytophilic and opsonic properties of immunoglobulins, and the way in which immunoglobulins cross the placenta or appear in external secretions.

My comments will be limited to focusing attention on Dr. Smithers' references to anaphylactic antibodies and Dr. Remington's to antibodies in external secretions.

The presence of antibodies in stools (coproantibodies) was first demonstrated in 1922, but the renewal of interest in these antibodies and their possible protective role is due to the recent demonstration that IgA is the predominant immunoglobulin in most external secretions, including the gastrointestinal secretions. It is likely that this IgA is a dimer of the serum monomer plus a protein "piece." The protein piece is found in epithelial cells and may be involved in transporting the IgA into the lumen of the gut. The probability exists that these IgA molecules are produced locally as a result of antigenic stimulation of the gut-associated lymphoid tissue by ingested antigens. Diseases in which various stages of protozoan and metazoan parasites reside in the intestinal tract provide opportunities for study of the importance of this system of local production of antibodies to parasite antigens in immunity against these infections. These diseases also provide models for fundamental studies of the immunological capacities and reactivities of the gut-associated lymphoid system.

Fixation of immunoglobulins to cells: Skin sensitization, anaphylaxis

We have only incomplete knowledge about which classes or subclasses of immunoglobulins fix to different types of tissue cells. Ideally, we should know the specific structural configuration of the heavy chain of each class of immunoglobulins that is responsible for its sensitization of (presumably by fixation to) different tissue cells.

The capacity to sensitize tissue cells is a property of antibodies essential for induction of the anaphylactic reactions that follow combination with antigen.

1. Reaginic skin-sensitizing antibodies (homologous anaphylactic antibody, homocytotropic antibody) are those antibodies that sensitize tissue cells (presumably mast cells) of their own or closely related species to produce systemic, local, and, under certain experimental conditions, *in vitro* anaphylactic reactions. In the human, these antibodies are detected by passively transferring serum into the skin of other humans (Prausnitz-Küstner reaction) or of certain primates, and allowing 24 hours or more to pass before injecting antigen to test for increase in vascular permeability. It has recently been shown that these antibodies pro-

duce *in vitro* sensitization of monkey ileum in a modified Schultz-Dale technique (1). The necessity for employing Prausnitz-Küstner reactions as an assay for this type of antibody has heretofore been one of the main obstacles to its characterization and purification. It is known to occur in the electrophoretically fast immunoglobulins (γ-1 mobility), to be present in very small amounts and to sediment in the ultracentrifuge faster than the bulk of the IgG molecules. Reaginic skin-sensitizing antibody sticks in the skin for weeks, although *in vitro* it is heat-labile and losses in activity occur with exposure to mercaptoethanol. Although thought for some time to reside in the IgA globulins, skin-sensitizing activity has now been reported in what may be a new class (IgE) of immunoglobulins (6).

Antibodies with similar reaginic skin sensitizing activity have been reported in the rat (4, 7), dog (14), and rabbit (13, 16). These antibodies are also found in trace amounts in the serum, persist in the skin for weeks after passive transfer within the species, are heat-labile, and are destroyed by mercaptoethanol treatment. The rat antibodies are reported to sediment faster than the bulk of the 7S antibodies. In rabbit they are reported to be 7S (13) or perhaps somewhat larger than 7S (16).

2. A second group of antibodies, also termed anaphylactic antibodies, with similar, but not identical, biological properties, and with different physicochemical properties, has been described in the guinea pig (3, 15) and in the mouse (2, 12). These antibodies also reside in an electrophoretically fast class of immunoglobulins, but this class, provisionally termed γ-1, is present in large amounts in the serum and has a sedimentation constant of 7S. In these two species, these γ-1 anaphylactic antibodies also mediate and transfer anaphylactic reactions within the species or in closely related species, but the skin-sensitizing activity is not destroyed as readily by heat or mercaptoethanol treatment and passively transferred antibodies persist in the skin for days rather than weeks (5, for review).

While it may be dangerous to make close comparisons between different animal species in which different immunoglobulin classes could well serve similar functions, it does seem to make sense from a phylogenetic point of view to look for both types of reaginic antibodies in both groups of species. It is interesting that Mota (8) has reported evidence of the existence of both types of anaphylactic (homocytotropic) antibody in the mouse, and I suspect that this will also be found to hold for the guinea pig. In the human, the rabbit, and the dog, on the other hand, where no discrete γ-1 has been reported, it is possible that the analogous immunoglobulin may turn out to be one of the four subclasses of IgG (12).

In any case, Johannson and Bennich (personal communication) have recently discovered a myeloma protein that does not react with monospecific antisera against IgG, IgM, IgA, or IgD and that has been found to have antigenic determinants in common with what has been termed IgE by K. Ishizaka (personal communication). If this myeloma protein has homocytotropic properties, the way may be open for chemical studies to relate a structural component of the heavy chain of the new immunoglobulin to the property of tissue (mast-cell?) sensitization. In addition, we need more studies of the kind Dr. Smithers described to learn whether the antibodies that produce anaphylaxis by virtue of their tissue-sensitizing properties may also play a role in immunity in the parasitic diseases. Certainly experimental infections with parasites appear to be an excellent measure for eliciting the production of these anaphylactic antibodies.

There is need for a better understanding of the functional significance of the heterogeneity of immunoglobulins in terms meaningful for better control of public health problems. This need and the potentially short step between advances in basic knowledge about the structure and function of immunoglobulins and the application of this knowledge to problems of public health are reflected in the development of the WHO immunology research program and the WHO Reference Laboratory for Im-

munoglobulins at the Institute of Biochemistry in Lausanne.

Victor Nussenzweig

In the ten minutes allowed me, I should like to discuss only two aspects of the preceding papers which are perhaps more controversial and might have important theoretical implications.

The first is the possibility that in many parasitic diseases in which there are high levels of immunoglobulins in the serum, these are nonspecific. The immunoglobulins would not be antibodies against the parasite or against its metabolic products or antibodies against cross-reacting antigens elicited through an anamnestic response. It is very difficult to understand the mechanism of such a nonspecific stimulation of γ-globulin production. Two possibilities come immediately to my mind. First, that the antigen specifically stimulates the immunocompetent cells. The plasma cells synthesize antibodies but simultaneously produce some nonspecific immunoglobulins. According to this hypothesis, one plasma cell would have to produce more than one kind of immunoglobulin, and this is not supported by most of the experimental evidence. Second, that during these infections there is a nonspecific stimulation of immunocompetent cells, and they proliferate and synthetize antibodies directed against products that have nothing in common with the antigens of the parasite.

Although I think these are interesting possibilities, there are no firm experimental data to support them, and at least in some cases other explanations can be found for this increased production of immunoglobulins. The situations in which very high "nonspecific" levels of serum immunoglobulin are found result, in general, from immunization with complex antigens. When well-defined substances are used as antigens, a substantial proportion of the serum immunoglobulins is very frequently found to be antibodies against the immunizing antigens. When some haptenic systems are used and the antibody production is studied at the cellular level, it can be demonstrated that during the first weeks after immunization as much as 50 per cent or more of the cells engaged in immunoglobulin synthesis are actually producing antihapten antibody (3).

It is also well known that many substances and bacterial products are potent immunogens in extremely small doses. If a minor parasite component or metabolic product can induce the formation of high levels of antibodies, the absorption of these antibodies with a mixture of different antigens would be very difficult if the important one constituted a minute fraction.

I should also like to bring to your attention some known facts on serological reactions in visceral leishmaniasis. This is one example of a parasitic disease in which extremely high levels of immunoglobulins are found in the serum. Many years ago I tried to obtain from cultured *Leishmania donovani* antigenic fractions that would react strongly and specifically with the immunoglobulins present in the patient's serum, but without success; this has been, I think, the experience of many investigators. However, when I used extracts of tubercle bacillus as antigen (as others had done before) I obtained very high complement-fixation titers with the patients' sera. Just to give you an idea of the results, in the complement fixation test I used .05 ml of the patients' serum and 6 $C'H_{50}$; in many cases the serum could be diluted 3,000 times and the results would still be positive (1). If this test is capable of detecting 1 μg/ml of antibody protein, some sera would contain about 3 mg/ml of antibodies against some components of the tubercle bacillus. The conclusion would be that a sizable portion of the immunoglobulins in the patients' sera have a well-defined specificity. It is fair, I think, to postulate the presence of cross-reacting antigens in *L. donovani* and tubercle bacillus. These antigens would perhaps be difficult to extract from the flagellates or be in some way altered during the extraction, while they could be easily obtained from tubercle bacillus.

The second general point I want to discuss is related to the problem of selection of immunoglobulin chains during the immune response. This is indeed an intriguing problem. A great number of different polypeptide chains are found among immunoglobulins, but a certain selection seems to take place during the immune response, and, as a consequence, antibodies may contain a restricted number of types of polypeptide chains. Why, for instance, are reagins produced in larger amounts in helminth infestations than in other infections? Why are certain antibodies preferentially located in one or other of the immunoglobulin classes? It is known, for example, that antipenicillin hemagglutinins or cold agglutinins are mostly IgM, while antidextran antibodies are IgG. In collaboration with Dr. Benacerraf, we have recently done some experiments that show that antihapten antibodies, containing certain classes of chains in preference to others, are synthesized by immunized animals because these chains are better fitted for the formation of the antibody-combining site (2). We have found that during the immune response of guinea pig to dinitrophenol (DNP) conjugates, a selection of types of L chains in the anti-DNP antibodies can be demonstrated. At the beginning of the immunization, when the binding affinities of the anti-DNP antibodies for ε-DNP-L-lysine are low, they contain both $\varkappa$ and λ types of L chain. However, when the binding affinities of the anti-DNP antibodies increase, only the $\varkappa$ type of L chain can be found. We have also found an increasing amount of L molecules (containing λ chains) in fractions of anti-DNP antibodies with decreasing affinities for the hapten. The implication would be, then, that $\varkappa$ chains are better fitted to produce anti-DNP antibodies of high binding affinity. This may be a general mechanism of selection of immunoglobulin chains during the immune response, and it would imply, for example, that the increased production of reaginic antibodies in some parasitic diseases is due simply to the fact that the H chain present in this kind of immunoglobulin is more adequate than other kinds of H chains to form "good" antibodies against some important parasitic antigen. It is possible, as was suggested by Dr. Bier, that some antienzyme antibodies play an important role in the mechanism of resistance in parasitic diseases and these antibodies might contain restricted types of H and L chains. Of course, a consequence of this reasoning might be that the reaginic antibodies that participate in the immune defenses mechanisms in some helminth infections are effective not because of their biological properties but because they specifically combine with some important antigen. This hypothesis can of course be tested experimentally, in view of the possibility of separating, by appropriate enzymatic treatment, the Fc portion of the antibody, which mediates its biological property, from the portion containing the combining sites.

I should like to finish with a word of caution about the assumption that IgM antibodies always precede the formation of IgG antibodies. I think that in many cases this issue has been confused by the differences in sensitivity of assays for those immunoglobulins. For example, Robbins, Kenney, and Sutter (4) have shown that rabbit IgM antibody to *Salmonella typhimurium* is 22 times more active than IgG in inducing agglutination, 120 times more potent in sensitizing bacteria for complement fixation, and 500 to 1000 times more efficient as an opsonin. Recent investigations using more adequate methods to detect antigen-antibody reactions show that in many cases IgM and IgG appear simultaneously after immunization.

Moderator: I should like to have the comments of Dr. Smithers and Dr. Remington on what has been said by the two discussants.

Smithers: This question of reaginic formation by helminth infection has interested us for a long time, and we have been very much puzzled as to why reagins are induced by helminth infections and only living helminth infections. The fact that they are not induced by dead worm homogenates but only by the living infection does suggest in fact that it is an antigen produced by the living worm—an excretion or secretion from the worm. I was greatly

interested in the suggestion of the last speaker that reagins may be induced by the stimulation from a particular type of antigen. In fact, we have found that allergens—that is, the antigens responsible for inducing reagins—can be produced best from a helminth by *in vitro* culture. We can produce a high level of allergen free in the culture fluid by maintaining schistosomes for four hours in Hank's solution, and the actual schistosome worms themselves show no depreciation in their allergen content, which suggests that they are actively producing allergen. The same applies even more to *Nippostrongylus*. If *Nippostrongylus* is incubated in buffered saline for three hours, two to three times as much allergen will be produced as if the adult worms were crushed.

And so it certainly looks as if the allergen were some sort of metabolic product. Indeed, some investigations have shown that these allergens are fairly small proteins, with a molecular weight of about 10 to 15 thousand, and they could well be, as the Moderator has suggested, an enzyme produced by the parasite.

The other small comment I should like to make is on Dr. Goodman's chart. The rhesus monkey could now be added to the human, dog, rat, and rabbit group, because it has been shown that rhesus monkey reagin is similar to those others. Dr. Sadun, I know, has found reagins in a chimpanzee infected with S. *mansoni*, and I am sure those would also be found to be the same.

Remington: I was very happy to hear Dr. Goodman's remarks about external body secretions. I think it is important to remember that the gastrointestinal tract is in the external world, not really inside our bodies, and that even in lower forms of animals, invertebrates, immunoglobulin similar to those of IgA are found; perhaps in prehistoric days this was one of the main modes of defense of such animals. We know very little about it in the human, and I think the parasitologists can help us learn more. I hope that some of you here, and your colleagues, will be interested in working in this area.

One comment about Dr. Nussenzweig's work: I was very much interested to hear about the antibody to the tubercle bacillus. As I am sure he is aware, if one wants to get a good deal of antibody against many antigens one finds a patient with cirrhosis of the liver who has hypergammaglobulinemia; his serum will contain large amounts of antibody. I wonder whether the mere finding of high titers to tubercle bacillus without adsorption of the immunoglobulins really shows that any of this hyperglobulinemia had anything to do with the tubercle bacillus, or whether this is just a simple manifestation of extra antibody being made in a hyperglobulinemic animal. Did you adsorb the gamma globulin portion with the tubercle bacillus to show a reduction in the total gamma globulin in those animals, Dr. Nussenzweig, and was this antibody present in the controls?

Nussenzweig: The reaction of kala-azar sera with tubercle bacillus has been known for a long time, and its sensitivity and specificity have been well studied. It has all the characteristics of a true antibody-antigen reaction. It is quite specific in the sense that these antigens do not react with sera from other types of leishmaniasis or with sera from patients with other diseases (except, of course, leprosy or tuberculosis) in which high levels of gamma globulin are found.

Moderator: I should like to comment on what Dr. Nussenzweig has just said. I should say that what you are really detecting in kala-azar is one of the antibodies whose production can actually be demonstrated, but there may be other antibodies produced at the same time that we haven't the right reagent to demonstrate.

On the other hand, the parallelism between the total amount of antibody and the high titer of complement fixation does not mean very much, because the amount of antibody necessary to give such high titers in the complement-fixation tests may not represent a considerable fraction of the total amount of gamma globulin. What I would like to know is the homogeneity or heterogeneity of the globulin produced in kala-azar.

Nussenzweig: I think I was misunderstood. I was not implying that all the gamma globulins found in the serum of patients with kala-azar is anti-tubercle-bacillus antibody. What is clear, I think, is that part of what is called "nonspecific" gamma globulin has a well-defined specificity.

Moderator: I understood you quite well, but I raised the point because you talked about the parallelism between the increase in gamma globulin and increase in complement fixation.

Remington: I do not want to press the point too hard, Dr. Nussenzweig, but I do not know what the data are to suggest that even a high proportion of the gamma globulins are formed against this tubercle bacillus extract. Have you adsorbed the gamma globulins to show that a significant percentage are directed against that antigenic component?

Nussenzweig: No, I have not.

Moderator: My remark about the activity was just to answer your question, because even if the serum is adsorbed the fact that there would not be a great reduction in gamma globulin would not be very enlightening; you may just adsorb a very small amount of gamma globulin that is responsible for the activity. The only assumption we can make is that probably many other antibodies are being formed, but it is just a hypothesis.

Borsos: In this connection I should like to cite some experiments. Dr. Richard Asofsky at NIH has used purified endotoxin as the immunizing antigen, and he measured the production of antibody in germ-free mice by bacteriocidal reactions that are exceedingly sensitive, as Dr. Bier knows so well from his complement-fixation experiments. He is capable of detecting 10^2 antibody molecules per ml, which makes this reaction a very sensitive test for antibody production. On the injection of the endotoxin, antibody is formed; there is an accompanying increase in gamma globulin content. However, as much as 80 per cent of the immunoglobulin produced contains no antibody activity against the endotoxin. These experiments demonstrate that there is indeed production of immunoglobulin that has no antibody activity to the particular antigen used. It might be argued that the principle of the original antigenic sin operates here, and that to some related antigens an anamnestic response was produced. This is hypothetical; there is no evidence for it. So I think we have to accept the fact that nonspecific gamma globulin can be produced in response to antigenic stimulation.

Kagan: I should like to interject a note of caution about some of these immunoglobulin experiments. It is very important to evaluate the assay methods for measuring immunoglobulins. Hemagglutination tests, for example, will detect IgM antibody much more readily than IgG, and if a hemagglutinating system is used to evaluate antibody levels, the result will be a biased evaluation of the amounts of IgM versus IgG antibody. In fact, Stavitsky has recently suggested that the hypothesis that IgM is produced first, followed by IgG, is not true; he believes they are produced at the same time, but detecting these antibodies depends upon the assay used for evaluation.

My other comment concerns Dr. Remington's work. Using specific immunoglobulin antisera in the fluorescent-antibody tests, he can detect acquired congenital toxoplasmosis by measuring IgM antibody production in the fetus. He can also detect IgG antibody in passive transfer of globulin from the mother to the fetus. This has a very practical use in determining whether what the infant has is an acquired infection or antibody obtained by passive transfer. We need studies like this for other parasitic infections. In our laboratory we are busy characterizng the various classes of immunoglobulins. They should be studied as they evolve during infection and their levels should be detected quantitatively. Recent studies of this nature have been published by Tobey and his group for malaria, and we need other such studies for other parasitic infections.

The third area that we are particularly interested in is the role of the various classes of immunoglobulin in our diagnostic tests. We have to evaluate the efficiency of various diagnostic serologic procedures in detecting these various types of antibodies. It may turn out

that one test has a higher sensitivity than another for detecting a certain class of immunoglobulin, and therefore serves as a better parameter of infection.

Moderator: So far we have discussed a few aspects of immunoparasitology connected with antigens and antibodies. Our next period will be devoted to cellular reactions and the effects of the immune response on the parasites. First, Dr. Soulsby.

Lymphocyte, Macrophage, and Other Cell Reactions to Parasites*

E. J. L. Soulsby

Introduction

Almost without exception, a marked cellular response is a characteristic feature of parasitic infections. Even a cursory examination of a supposedly simple reaction will reveal its complexity, and when a more major reaction is examined an evaluation of it would seem to be an extremely hazardous enterprise. Fortunately, the newer knowledge of the various cell types will now permit a closer study of the functional aspects of the cell response, and it is this aspect which will be emphasized. A familiarity with the morphology and origin of the cells will be assumed.

The chief concern of this review will be the cell types that are closely associated with the immune response of the host, those that respond as a result of it and other forms that make up the acute inflammatory response. No attention will be paid to degenerative changes that occur in parenchymatous cells of organs or proliferative changes in, for example, fibroblasts, but the newer knowledge about the change or loss of function of epithelial cells in parasitic infection warrants brief consideration.

Epithelial cell reactions to parasitism

Hyperplasia of epithelial cells is a common response to parasitism. It is seen in the bile duct or pancreatic duct when these are parasitized by helminths such as *Fasciola, Clonorchis, Ascaris, Hymenolepis,* and *Stilesia* or by protozoa (e.g., *Eimeria stiedae*). Similar responses occur in the urinary bladder (polyp formation) in schistosomiasis, in the bronchioles and bronchi in lungworm infection, and in the gastrointestinal mucosae in nematode, trematode, and sporozoan infections. Such changes are frequently accompanied by a loss of function of various specialized cells or their acquisition of new functions. For example, in *Ostertagia* infection in the abomasum of sheep and cattle, the specialized parietal cells that produce hydrochloric acid and the peptic cells that produce pepsinogen may lose their function and be replaced by hyperplastic undifferentiated cuboidal cells (51). In the same infection, mucoid metaplasia may occur, and in fact this reaction—seen in the bile duct, in the stomach mucosa, in the bronchi, and elsewhere—is a not uncommon response to parasitic helminths.

Recent investigations have correlated such changes with the molecular biology of the parasitism. In the *Ostertagia* situation, the loss of cells with specialized secretory function leads to a rise in the pH of the abomasum and a failure to activate pepsinogen, a leakage of pepsinogen into the blood, and an increased leakage of plasma macromolecules into the lumen. The last two of these effects may be due to imperfectly formed cell junctions as a result of the hyperplasia (51).

The functional changes in the rat intestinal epithelium, which becomes hyperplastic in *Nippostrongylus braziliensis* infection, have been studied by Symons and Fairbairn (101). Under normal conditions, it is said, there is a

* The experimental work reported in this review was carried out with the support of USPHS Grant AI 06262.

progressive differentiation of the function of epithelial cells as they migrate distally to be shed at the tips of the villi (83). *N. braziliensis* infection appears to accelerate this migration, leading to an immaturity of the cells with a concomitant loss, or reduction, of the levels of maltase, alkaline phosphatase, and leucine amino peptidase on the cell surface and in the microvilli of the epithelial cells. Comparable hyperplastic changes have been reported in *Necator americanus* infection (94), and are said to contribute to impaired absorption of vitamin A, xylose, and fat.

Though there is much to indicate that such changes are essentially nonspecific, being seen in nontropical sprue and niacin deficiency, they are associated with cell hyperplasia in the lamina propria, and this in parasitic infections includes infiltration of polymorphonuclear leukocytes, eosinophils, macrophages, plasma cells, and lymphocytes, the latter in various stages of transformation.

Local accumulation of lymphoreticular cells

Lymphocytes, plasma cells, and macrophages are traditionally associated with immunological functions, and in many instances the local accumulation of them may progress to a definite, and at times macroscopic, focus of lymphoreticular elements that has the general appearance of a lymph node. Such structures frequently develop around a parasite or its larval stage that has been trapped in the tissues. They are seen later in the course of an infection and frequently at a time when immunity (*sensu stricto*) develops. It is difficult to avoid the conclusion that these lesions are related to the mechanism of immunity, and in fact there is often much to indicate that immunity against a parasite is mediated at a local level. This statement would not, of course, imply that the major antibody-producing organs such as the spleen and lymph nodes do not contribute to the picture.

The local nature of the immune response at an organ level is well illustrated by bovine trichomoniasis, in which protective immunity appears to be mediated solely in the uterus and vagina. A similar situation possibly occurs also in *Trichomonas vaginalis* in man. This implies that antibody-producing cells are located in the uterine and vaginal walls, and in fact accumulations of plasma cells have been found there (88). Furthermore, it seems that the vagina can produce antibody independently of the uterus and of the general antibody-producing organs (87).

Another example of a locally mediated immune response, with local cell accumulations, occurs with *Eimeria tenella* of the cecum of the chicken. In this case, however, immunity created in one cecum is somehow transferred to a surgically isolated collateral cecum (45). The mechanism is not yet fully understood, though the transfer does not appear to be mediated by serum antibodies (46). Pierce and Long (88) suggest that the immunity that develops at a second, previously unstimulated site (the second cecum) may be analogous to the second-set homograft rejection reaction (65).

Accumulations of lymphoreticular cells in helminth infections are seen perhaps to their best advantage in such entities as the lmphoreticular broncho-occlusive lesions in lungworm disease of cattle that occur around larvae trapped in the tissues or eggs and larvae that have been aspirated into bronchioles and alveoli (53) and also in schistosomiasis with the formation of pseudotubercles around immature schistosomes and eggs in the liver.

Lymphoid hyperplasia in Leishmania infections

The immunological role of lymphoid cells in Old World cutaneous leishmaniasis seems fairly clear and has been documented by Adler (1, 2). After infection with *Leishmania tropica,* there is a local proliferation of macrophages in which the leishmaniae multiply. This continues until the area is infiltrated with lymphocytes and plasma cells; when this happens the macrophages cease to proliferate, the population declines, and the number of parasites also decreases until they can be demonstrated by culture techniques only. Eventually the orga-

nisms disappear completely and the cutaneous lesion resolves. The sequence of events varies with the individual and in the absence of specific therapy may take three to eighteen months. Spontaneous cure is followed by lasting immunity to the causative strain, which may persist for as long as 20 years. Immunity is established only after the lesion has progressed through the series of cellular reactions that result in spontaneous cure (1); if the cutaneous sore is removed surgically before spontaneous cure, the individual remains susceptible to reinfection.

A similar situation occurs with certain forms of New World cutaneous leishmaniasis. The Uta of Peru is usually followed by immunity; however, with mucocutaneous leishmaniasis, though the initial cutaneous lesion usually heals spontaneously, metastatic lesions occur in the skin, the mucosa of the mouth, and the cartilages of the nose, mouth, and nasopharynx. A subsequent lymphocytic and plasma cell infiltration may reduce or eliminate the parasites, but in the mucocutaneous form of the disease chronic metastatic lesions continue to occur. A common feature of these forms of cutaneous leishmaniasis is the lymphoid infiltration, accompanied by a positive Montenegro skin reaction of the delayed type . The reaction develops early in the course of infection and persists long after spontaneous cure. It can be induced by leptomonads of *L. tropica* or other species of *Leishmania,* including those of cold-blooded animals, and also antigens from *Trypanosoma cruzi* and antigens of *T. equiperdum* (32).

A type of leishmaniasis in which there is little or no lymphoid cell invasion and no positive Montenegro reaction is *Leishmaniasis tegumentaria diffusa* (19). In this, extensive areas of skin are involved and masses of infected macrophages are found in the dermis, with no secondary invasion of lymphocytes or plasma cells. It has been suggested that the condition, which has been recorded in a small number of persons in Bolivia, Venezuela, and Brazil, may be one of immunological unresponsiveness on the part of the infected person (1). However, no work has been done to determine whether such persons are genetically deficient or whether the unresponsiveness is one induced by the parasite.

The immunological response to cutaneous leishmaniasis would suggest that it is mediated by delayed-hypersensitivity mechanisms. Circulating antibodies are not readily demonstrated in the infection, and there is no evidence that the immunity has an antibody basis.

The situation with visceral leishmaniasis is quite different from that of the cutaneous form. The cellular reactions are similar, characterized by a massive proliferation of histiocytes and secondary infiltration of lymphoid elements generalized throughout the spleen, liver, bone marrow, and lymphatic glands. However, in most untreated human infections the disease is fatal, though spontaneous cure may occur—up to 25 per cent of such cases have been reported in India (71) and in Portugal (32). Therapeutic cure of kala-azar is followed by resistance to reinfection, and in about 10 per cent of these a local skin lesion (post-kala-azar dermal leishmanioid) may develop. Though this lesion contains numerous parasites, they do not become generalized but rather appear to represent a residuum of infection for the maintenance of immunity or premunition.

Whereas delayed skin reactions are common in dermal leishmaniasis, active cases of Indian kala-azar do not show a Montenegro reaction (93) and an absence of the reaction has been noted in Brazilian cases of kala-azar (3). A similar situation appears to obtain in the Mediterranean form of the disease. However, some cases with post-kala-azar dermal leishmanioid do give a positive Montenegro reaction (2). The situation appears different in the East African form of kala-azar: positive Montenegro reactions have been observed in treated infections and in 95 of 119 normal volunteers inoculated with a gerbil strain of *Leishmania* that localizes in the dermis (2).

The functional value of the lymphoid hyperplasia in untreated visceral leishmaniasis is difficult to evaluate. Basically, it fails to contain the infection, but there are indications that the vigor of the lymphoid response may have some value in prolonging life in man and dog. On the other hand, spermophils are very susceptible

to infection and show excessive macrophage proliferation with less lymphoid cell infiltration than man or dog. In the spermophil the spleen may be converted to "a nearly solid histiocytoma" (2).

Specific antibodies for *Leishmania* have been detected in visceral leishmaniasis by complement-fixation techniques, either by the use of leishmania antigens or by extracts of mycobacteria, the former being more satisfactory for this (19). There is, however, little correlation between the elevated levels of gamma globulin and the development of immunity or between the complement-fixation tests and immunity.

Lymphoid hyperplasia in Theileria infections

The *Theileria* genus occurs in ruminants, and though a detailed consideration of the cell reactions in this infection is not germane to the discussion, a brief consideration of the immune response serves to illustrate an infection in which immunity, when it does occur, is solid for many years. The important species in *Theileria parva,* which causes East Coast fever, a disease that is usually fatal and is characterized by lymphoid hyperplasia followed by exhaustion of the lymphoid tissues and leukopenia (48). Immunity cannot be reduced by splenectomy, and its level is not influenced by the degree of clinical response to the first infection. Antibodies have not been regularly detected in infected or immune animals (9).

In this infection, however, the lymphoid cells appear to play a dual role: besides their presumed importance in the immune response, they also serve as host cells for the parasites. Recent work has shown that the two replicative forms of the parasite, the "macroschizonts" and "microschizonts," behave differently in lymphoid cells (48). The former has been cultivated in bovine lymphocytes in association with baby hamster kidney cells, and in these the organism propagates in the multiplyng lymphoid cells but does not destroy the host cell (47). The *Theileria* organism appears to divide at the same rate as the host cell, the parasitic forms being closely associated with the mitotic apparatus and distributed to daughter cells in late mitosis. This process has yet to be conclusively demonstrated *in vivo,* but so far there is no evidence for new infection of cells by particles liberated from distintegrated lymphoid cells.

It has yet to be determined whether the parasitized lymphocyte or its clonal descendants can eventually become immunologically competent or whether a separate line of cells is involved in this process.

Lymphocyte populations and immunoglobulin types in parasitic infections

It is only recently that efforts have been made to determine the relative proportions of immunoglobulin-containing cells found at the local site of an immunological event in a parasitic infection. Recent studies (26) of rabbit tissues during experimental trichinosis have used pairs of immunofluorescent reagents specifically reactive with the γ-, μ-, and α-heavy chains and labeled with contrasting fluorochromes (17).

A preponderance of IgA-containing cells was found in intestinal sections, but this was high both in normal and in infected rabbits and cells containing the α chain made up 80 to 90 per cent of the immunoglobulin-containing cells in normal animals. A comparable finding has been reported for the human intestine (24). A relative increase in IgM-containing cells was observed early in *T. spiralis* infection, followed by an increase in IgG-containing cells late in the infection and after hyperimmunization. The distribution of the immunoglobulin-containing cells in the spleen and popliteal lymph nodes differed from that in the intestinal mucosa, with IgA cells constituting only 2 to 10 per cent of the fluorescing cells. Soon after infection the proportion of IgG cells to IgM cells was relatively high, but late in the infection and in hyperimmunized animals the proportion was usually reversed.

In the diaphragm, after larval encystment, each type of immunoglobulin-containing cell was observed; the distribution was similar to

that in the spleen, and IgM cells were the most abundant.

The role of IgA cells in immunoglobulin production in the intestinal mucosa and in immunity to parasites in general has yet to be clarified. Crandall *et al.* (26) failed to observe fixation of IgA immunoglobulin to *T. spiralis* larvae when sections of diaphragm containing larvae were exposed to various immunoglobulins. Specific staining was obtained only with anti-γ chain reagent.

Interest in IgA antibody has increased recently because of the demonstration of anaphylactic antibody in rats infected with *N. braziliensis,* in monkeys infected with *Schistosoma mansoni,* and in other parasitic infections (78). Though not all the anaphylactic antibody detected in these infections may belong to the IgA type, the occurrence of IgA-containing cells at the site of an immunological event, especially when anaphylactic mechanisms are postulated as mediators of the immune response, may indicate that these cell types are important in the response. The situation in *N. braziliensis* infection may, however, require some reconsideration in the light of recent work with neonatally thymectomized rats, in which strong resistance developed in the absence of high levels of anaphylactic antibody (113).

Effect of immunosuppressive agents on immunity to parasites

The manipulation of the immune response to parasites by immunosuppressive drugs, irradiation, thymectomy, and bursectomy has been little studied to date. It should, however, offer an invaluable tool in the analysis of immunity to parasites.

The adrenal steroids have been used in a variety of studies on immunity to parasites, and it has been demonstrated, for example, that the elimination of adult worms of *Trichinella spiralis* in mice, probably an immune event, can be markedly inhibited by cortisone (20, 58). In sheep infected with gastrointestinal nematodes, excessive doses of adrenal steroid (prenidsolin) failed to have any effect on the immune status (Soulsby, unpublished), but chlorambucil markedly affected immunity and allowed a population of inhibited larvae to attain patency within a few days (100). On the other hand, prenidsolin has been used successfully to inhibit the immune elimination of *N. braziliensis* from the gut of rats (77) and cortisone has been used to overcome "innate" resistance to such helminth parasites as *Litomosoides carinii* (15) and *Nematospiroides dubuis* (28).

Interpreting the effects of adrenal steroids on immunity to parasites is difficult. These compounds have a wide range of effects on almost every aspect of the immune response (see 40 for review), and consequently it would be unwise to infer a common basis for the immunity from a common end effect of the drugs.

Total body irradiation has been used to study immunity to *T. spiralis* infection, and exposed mice failed to show a significant elimination of their adult worms as compared with control animals (115). As might be expected, the irradiation produced a severe leukopenia, but circulating antibody levels were not markedly altered over the experimental period.

Studies on the effect of bursectomy and thymectomy on immunity to the chicken coccidian *Eimeria tenella* were carried out by Pierce and Long (88). Chickens deprived of bursal tissue by *in ovo* treatment with testosterone were successfully immunized against *E. tenella* even though they failed to produce serum antibodies and showed markedly reduced or undetectable levels of immune globulins. In addition, pyroninophilic cells in the ceca or spleen and secondary foci in the spleen and cecal lymphoid tissue were also very much reduced in numbers. The inhibition of bursal development is recognized as a major factor in reducing the ability of fowls to synthesize immunoglobulins; nevertheless, such fowls are still able to reject skin grafts (108). The indication is therefore that immunity to *E. tenella* is mediated more by cellular elements than by humoral antibody. Unequivocal evidence that immunity to *E. tenella* in the chick was dependent on cells derived from the thymus was not obtained, but this was probably due to the difficulty of ensuring that all thymic tissue had been removed. In any case,

thymic tissue is present in chicken embryos after 14 days of incubation, and there is the possibility that lymphocytes from the thymus had already been distributed in the body by the time of hatching (85).

The effect of thymectomy on immunity in the rat to *N. braziliensis* has been reported, and in this work neonatal thymectomy failed to alter the acquisition of immunity to the parasite (113). It is of interest also that thymectomy caused a marked reduction in the level of anaphylactic (PCA) antibody.

Much of the foregoing evidence repeatedly invites the idea that in many cases immunity to parasites is mediated more by "cellular immunity" than by classical humoral antibody. There is, indeed, increasing justification for this belief in respect to some infections, but it should not be forgotten that other entities (such as malaria, trypanosomiasis, and larval cestode infections) do appear to depend on humoral factors for the protective immune response. In fact, it should be no surprise to find that a whole range of immune responses occurs to parasitic infection and that the protective devices employed vary from parasite to parasite.

Relationship of delayed hypersensitivity (cell-mediated immunity) to immunity to parasites

In many parasitic infections there has long been an inability, or controversy about the ability, to passively transfer immunity with serum. In cases where this has been achieved, comparatively large volumes of serum have been required and frequently only a moderate degree of passive immunity has been achieved. Local passive transfer of antibody into the skin, with subsequent challenge of the sensitized site with cercariae, has been used to demonstrate serum transfer of immunity in schistosomiasis (79). On the other hand, protective immunity has been transferred by lymphoid cells in at least two nematode infections in which serum transfer failed to convey immunity: with lymph node cells from guinea pigs infected with *Trichostrongylus colubriformis* (34, 107) and with peritoneal cavity cells with *Trichinella spiralis* (58). A recent report has indicated that serum or lymphoid cells, or the two together, could transfer immunity to *Ancylostoma caninum* in dogs (66). Larsh (58) has gone as far as to conclude that the mechanisms causing the expulsion of adult *T. spiralis* in mice are mediated by a specific delayed hypersensitivity reaction. Hypersensitivity of the delayed type to larval antigens of *T. spiralis* has been demonstrated following the injection of antigens with Freund's complete adjuvant into the foot pad of guinea pigs (56).

Delayed hypersensitivity is well known in leishmaniasis and is the basis of the Montenegro skin reaction for the diagnosis of mucocutaneous leishmaniasis. It is of interest to note that the leishmaniae are intracellular parasites of macrophages, and in a recent review of delayed hypersensitivity and microbial infection, Mackaness (62) has stated that "without any known exception, organisms which can survive and multiply within host macrophages cause delayed-type hypersensitivity to . . . the microbial antigens." A feature of microbial infections in which delayed hypersensitivity plays a pronounced part is that immunization with killed vaccines (except for *Mycobacterium*) usually does not lead either to a delayed hypersensitive response or to marked protection against the challenge infection (62). Living vaccines, on the other hand, produce both. The similarity between this situation and that seen with a number of parasitic infections is striking, and it would be all too tempting to ascribe many of the difficulties in understanding immunity to parasites, and to helminths in particular, to delayed hypersensitivity phenomena. There are no adequate data at present, however, to support any such claim, though there are indications in several directions of a closer relationship between the delayed type of hypersensitivity and parasite immunity than has hitherto been suspected.

A major objection to such an idea might be that skin reactions of the delayed type have not been regularly observed in parasitic infection. However, a delayed skin response is seen in *Leishmania*, *Toxoplasma* (39), *T. cruzi* (63),

and the early stages of infection with a number of helminths. It is possible also that the delayed skin reaction has not been searched for, especially when an infection already has a marked immediate-type response. A further point is that a skin response may be only one of several manifestations of delayed hypersensitivity and its absence may imply nothing about the reactions occurring at a cellular level.

Mackaness (62) has suggested that continuing antigenic stimulation is necessary for the induction of acquired cellular resistance. Such a situation could certainly obtain in parasitic, especially helminth, infections where materials may persist in the tissues for several months.

In any consideration of specific cell-mediated immunity, the lymphocyte plays a prominent part. Cells that derive from small lymphocytes, pyroninophils, or "immunoblasts" (29) are very much in evidence in the lymph nodes draining a skin homograft, at the rejection site itself, and in lymph nodes draining a site to which a contact sensitizing agent has been primarily applied. If searched for, such cells are also common in the local sites of an immune event in a variety of parasitic infections.

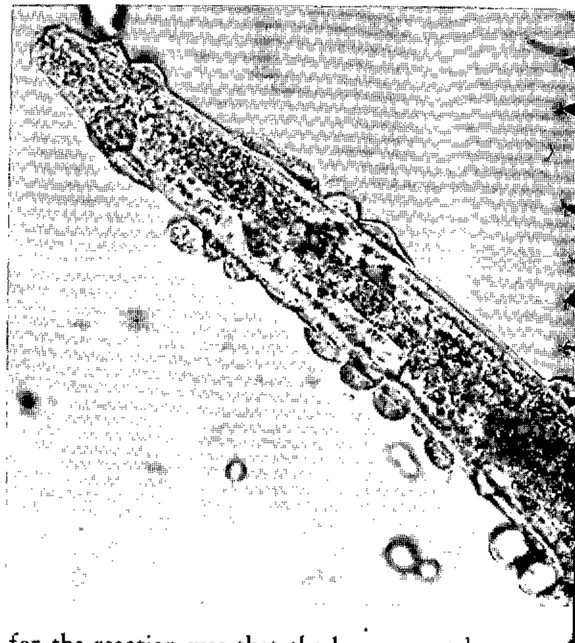
Hitherto, a major problem in the study of delayed hypersensitivity has been the absence of an *in vitro* correlate of the condition. The situation is rendered more difficult in the field of parasitology because of the lack of suitably defined antigens. Recently, however, several *in vitro* and experimental *in vivo* systems have been suggested as *in vitro* correlates, including the inhibition of migration of macrophages from capillary tubes, the transformation of small lymphocytes to active blast forms by soluble antigen (or homologous or heterologous lymphocytes), and the disappearances of macrophages from the peritoneal cavity on the injection of antigen. Of less certain significance is the antibody that is cytophilic for macrophages.

A reaction that might, after further study, serve as an *in vitro* correlate of immunity in helminth infections is one in which pyroninophilic lymphoid cells become strongly adherent to the antibody-sensitized surface of helminth larvae.

Interaction between pyroninophils and parasites

Original studies on this system were concerned with the *in vivo* interaction of *Ascaris suum* larvae with cell populations in the peritoneal cavity of immune rabbits (97). It was found that within one to four hours third-stage larvae became covered with a mass of cells, which, when stained, were seen to be a mixture of cells with a distinct pyroninophilic cytoplasm and eosinophils. The reaction could be followed *in vitro* with a peritoneal cell exudate induced by a bland oil. The adherence of cells to larvae was rapid and firm. An essential requirement

FIGURE 1. Cells from peripheral blood culture adherent to third-stage *Ascaris* larvae (x 350).



for the reaction was that the larvae must have been previously sensitized with antibody; the treatment of larvae with normal serum failed to induce the reaction. The most reactive cell populations were those containing a high proportion of lymphoid cells; exudates consisting principally of macrophages produced poor reactions or none at all. Since the major reacting cell appeared to be of the lymphoid origin, the reactivity of cells from various lymphoid organs was examined.

Cell suspensions in Eagles Minimal Essential Medium (MEM) plus 5 per cent normal rabbit serum were prepared from popliteal and mesenteric lymph nodes and the spleen of normal rabbits and of rabbits immune to *A. suum*. Lymphocytes from lymph nodes failed to become adherent to the antibody-sensitized surface of *A. suum* third-stage larvae, and when such lymphocytes were exposed to anti-*A. suum* serum they similarly failed to adhere to larvae.

Slight adherence of cells was seen with splenic cells from immune rabbits but not with cells from normal rabbits. The cell adhesion was of a low order, however, and not comparable to that observed with peritoneal cell exudates. Exposure of spleen cells to anti-*Ascaris* serum failed to increase the degree of adhesion or cause adhesion with spleen cells of normal rabbits. A more detailed study of the reactive cells in peritoneal exudates suggested they were transformed lymphocytes, and in further work cultures of peripheral white blood cells stimulated either with phytohemagglutinin (PHA) or with *Ascaris suum* antigen were used. The cell cultures were prepared from heparinized blood obtained by cardiac punctures and cultured in MEM Spinner medium, with the addition of 20 per cent inactivated horse serum in "French Square" bottles. Each culture consisted of 10^7 small lymphocytes in 10 ml of medium, and to each was added either 1 per cent PHA or a total of 0.6 mg of protein of whole adult worm extract of *A. suum*. Cells were harvested after one, two, three, four, and five days of culture, and the cell suspension was centrifuged and washed three times in ice-cold MEM plus 5 per cent NRS and finally made up to 1/10 the original volume (1 ml). Third-stage *A. suum* larvae from culture were washed three times in veronal buffer and then sensitized to varying dilutions of antibody. Next they were washed three more times to remove unattached and unwanted serum proteins. White cell adherence reactions were examined for by mixing one drop of sensitized larval suspension with one drop of cells on a slide.

White blood cells from one- or two-day-old cultures failed to become adherent to the surface of antibody-sensitized third-stage larvae. The exposure of such cells to immune serum, with subsequent washing, also failed to induce cell adhesion. Stained samples of the cell suspension showed small lymphocytes, neutrophils, eosinophils, and macrophages. Cell suspension from three-day cultures contained cells that adhered strongly to the surface of antibody-sensitized larvae, and the degree of white cell adhesion increased with cell suspensions from four-, five-, and six-day cultures. After six days of culture, a marked reduction in the number of cells occurred.

Cell cultures aged three, four, and five days showed a decreasing number of surviving neutrophils and an increasing number of typical transformed lymphocytes or blast cells. These showed an expanded nucleus, distinct nucleoli, and a varying-sized rim of basophilic cytoplasm, and methyl green pyronine staining revealed a markedly pyroninophilic cytoplasm. Stained preparations of larvae with adherent cells showed the cells to be comparable to the blast forms in the culture, possessing a marked basophilic and pyroninophilic cytoplasm. Larvae with adherent cells were fixed and exposed to goat anti-rabbit globulin serum conjugated to fluorescein isothiocyanate (FITC) and examined under ultraviolet light. Strong fluores-

FIGURE 2. Pyronine stain of adherent cells on third-stage *Ascaris* larvae.

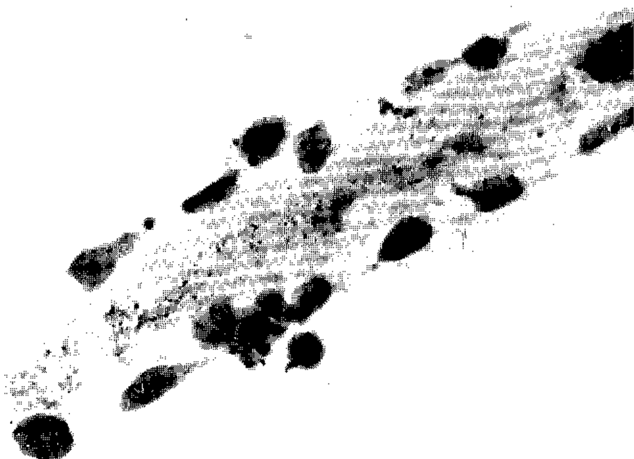
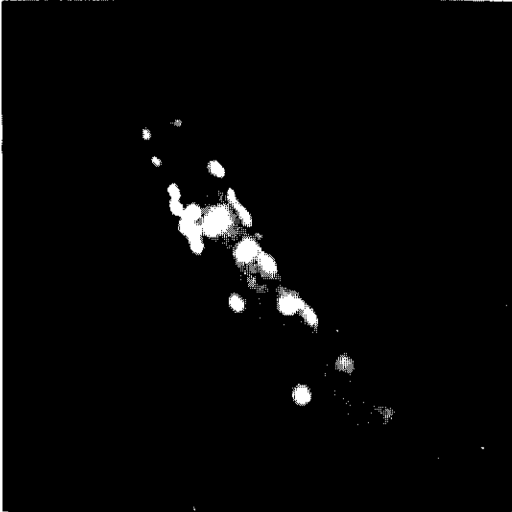


FIGURE 3. Cells on *Ascaris* larvae stained with goat anti-rabbit globulin serum conjugated with FITC.



cence occurred in the cytoplasm of the attached cells. Similar preparations of larvae and cells were exposed to *A. suum* antigen and after adequate washing were then exposed to a rabbit anti-*A. suum* globulin conjugated to FITC. In this case strong fluorescence occurred both in the cells and on the surface of larvae, giving presumptive evidence that antibody to *A. suum* occurred both on the surface and in the cytoplasm of the attached cells.

The reaction showed evidence of specificity in that the most marked reactions occurred with cell cultures obtained from rabbits that were strongly immune to *A. suum*. These had been immunized by repeated subcutaneous injection of infected eggs (96) or by vaccination with cultures of third-stage larvae obtained from culture (97). Cultures of cells from rabbits immune to unrelated antigens and stimulated with the appropriate homologous antigen failed, with one exception, to give strong lymphoblast adhesion (see Table 1). The significance of the reactions with cells from rabbits immune to egg albumin is unclear.

At low dilutions of immune serum, sensitized larvae attracted cells with equal effect from cultures stimulated with either antigen or phytohemagglutinin; however, with increasing dilutions of antiserum the cells from PHA-stimulated cultures were less reactive, and serum could be diluted to a point at which PHA-stimulated cells were nonreactive whereas antigen-stimulated cells still gave good leukocyte adhesion. Such results might be explained on the ground that the mitogenic effect of PHA stimulated the transformation of lymphocytes "committed" to a wide range of antigens, including *Ascaris*, but the number of cells in suspension specifically committed to *Ascaris* would be much smaller than in suspensions of lymphocytes stimulated by the *Ascaris* antigen.

The system was antibody-dependent, and the most satisfactory sensitization was achieved with serum from rabbits immune to *A. suum*. Thus, rabbits that had been immunized repeatedly with *A. suum* eggs (96) or with antigens prepared from *in vitro* cultured larvae (97) provided reactive sera, and the increasing ability of serum to sensitize larvae to attract cells could be titrated during the immunization schedule. Normal rabbit serum failed to sensitize larvae, and minimal sensitization was obtained with sera from rabbits immunized with various tissues of adult *Ascaris* (whole worm, cuticle, testes, and so on). No reaction was obtained when third-stage larvae were sensitized with immune serum against human blood group A substance, sheep red blood cells, fowl red blood cells, *Necator americanus*, or *Turbatrix aceti* (Table 2). In all these cases, sensitization of the cuticle by antibody could be demonstrated either by mixed antiglobulin agglutination (22), mixed agglutination, or immune adherence techniques (98).

The complement requirements for the reaction have yet to be fully clarified. Some sera retain their sensitizing ability after inactivation at 56° C for 20 or 30 minutes, whereas others fail to do so. The reactivity can be partially restored by fresh guinea pig sera and more or less completely restored with normal rabbit serum. The sera that are not inactivated by healing are, invariably, those that show the greatest sensitizing ability and are still active when inactivated at 56° C for 60 minutes, when treated with zymosan or ammonia, or when absorbed with sheep red-blood-cell stroma

TABLE 1. Leukocyte adhesion with antibody-sensitized *A. suum* larvae and cultures of blood lymphocytes from rabbits immune to different antigens and stimulated with the homologous antigen

ANTIBODY DILUTION USED TO SENSITIZE LARVAE	LYMPHOCYTES FROM RABBITS IMMUNE TO							
	Ascaris	*Ascaris*	*Ascaris*	Normal rabbit	Sheep serum	Bovine gamma globulin	Bovine serum albumin	Egg albumin
1 in 5	+ + +	+ + +	+ + +	+	+	+ +	+ +	+ + +
1 in 10	+ + +	+ + +	+ + +	−	−	+	+	+ + +
1 in 20	+ +	+ + +	+ +	−	−	−	−	+ +
Diluent	−	−	−	−	−	−	−	−

+ + + Strong leukocyte reaction
+ + Medium reaction
+ Weak reaction
− No reaction

sensitized with a rabbit anti-sheep-red-blood-cell serum. However, inactivation at 56° C for 120 minutes greatly reduced or abolished the sensitizing ability. Treatment of sera with 2-mercaptoethanol abolished the sensitizing ability, though such sera were still able to sensitize the surface, as determined by the mixed antiglobulin agglutination reaction. Samples of reactive sera were electrophoresed in agar, and various fractions were eluted from blocks cut from the electrophoretic run. Sensitizing ability for cell adhesion was found to lie in the region where one would expect the macroglobulin fraction of serum, but other serum fractions were able to sensitize larvae without causing leukocyte adhesion.

The significance of this reaction with third-stage larvae in the *in vivo* immune response remains to be determined. The present evidence indicates that with such larvae the reaction is largely mediated by cells and serum from animals immune to the parasite; however, further work is necessary before it can be claimed that the reaction is an *in vitro* correlate of protective immunity. It is of interest, however, that the reaction occurs only when sufficient time has been allowed for the small lymphocytes to be activated from their "resting" state.

The adherence to the surface of third-stage larvae of cells provisionally classified at present as transformed small lymphocytes is one of a range of white cell reactions with parasites. With the *A. suum* system it can be demonstrated that cells of the granulocytic series also become adherent to the surface of larvae, but this reaction lacks the marks of specificity that

TABLE 2. Leukocyte adhesion with *A. suum* larvae sensitized with various dilutions of different immune sera and tested with cultures of blood lymphocytes from a rabbit immune to *A. suum* and stimulated with *A. suum* antigen

ANTIBODY DILUTION	LARVAE SENSITIZED WITH SERUM FROM RABBITS IMMUNE TO							
	Ascaris vaccinated eggs	*Ascaris* vaccinated larvae	*Ascaris* whole worm	*Ascaris* adult cuticle	*Ascaris* adult testes	Blood group A_1	Sheep RBC	Fowl RBC
1 in 5	+ + +	+ +	+	+	+	−	−	−
1 in 10	+ + +	+ + +	−	+	−	−	−	−
1 in 20	+ + +	+ + +	−	−	−	−	−	
Diluent	−	−	−	−	−	−	−	−

+ + + strong leukocyte adhesion
+ + medium reaction
+ weak reaction
− no reaction

characterize the response with lymphoid cells. The reactions with granulocytes, together with other reports on the interreaction between parasites and white cells, will be discussed in the section dealing with neutrophils and eosinophils.

Lymphocyte transformation by parasite antigens

The transformation of small lymphocytes into larger blast forms able to synthesize DNA and undergo mitosis can be induced by mitogens such as phytohemagglutinin (75), by various antigens to which the donor is sensitive (such as tuberculin) (86), and by mixed leukocyte cultures (7). As far as can be determined, there has been little or no work using this technique for parasitic infections, but there would seem to be every justification for study in this direction in view of the lymphoid hyperplasia frequently seen in parasitic infection.

Preliminary experiments at the author's laboratory have indicated that lymphocyte transformation can be induced in rat lymphocytes derived from animals immunized with *A. suum* and stimulated with an extract of whole adult worm. Table 3 presents some of these preliminary results. Similar reactions have been obtained with peripheral blood lymphocytes of a man sensitive to *Ascaris* as the result of laboratory contact over many years (Table 4).

The significance of these preliminary findings is not yet clear. They may indicate that a delayed hypersensitivity component is a part of the immune response to *Ascaris* infection, and this would be in line with certain aspects of the histological picture of the immunity to *Ascaris*. However, it is certainly not the sole form of hypersensitivity that occurs, since an immediate type of hypersensitivity is also much in evidence (100). Nevertheless, it is possible that both could exist in the same animal, perhaps mediated by different antigens. The system does, however, provide a useful technique for the study of lymphocytes from different sources using small quantities of antigen, and the recent demonstration by Oppenheim *et al.* (82) that delayed hypersensitivity in the guinea pig is expressed by increased lymphocyte transformation should encourage work with *Ascaris* in the guinea pig.

Macrophages

Reticulo-endothelial hyperplasia has already been noted in leishmaniasis, the macrophage serving as a host cell for the parasite. Infected macrophages are very active and are able to penetrate into many tissues and appear to exchange material with neighboring cells by means of pseudopodia through which material passes from one cell to another (1). The general failure of the immune response in visceral leishmaniasis has been suggested as due to an equivalent of blockade of the RE system. However, the fact that the macrophages are actively phagocytic—indeed, they show increased indiscriminate activity—has been presented as evidence against immunologic paralysis (2), but this may not be a valid consideration, since it has been shown that the macrophages of rabbits rendered tolerant in neonatal life to BGG can take up antigen and stimulate DNA synthesis in spleen cells of an immunized animal (44). The absence of an obvious defect of recognition is comparable to the results of *in vivo* studies with bacterial antigens showing that tolerant animals were able to recognize antigen in the same way as immune animals (74).

The phagocytic function of the macrophage is of particular interest in the hemoprotozoa, and it is now clear that erythrophagocytosis is a major factor in the pathology of malaria (115). Extensive erythtrophagocytosis of both parasitized and normal erythrocytes is seen in the internal organs in *Plasmodium falciparum* infection and has also been observed in *P. berghei* in rats (23) and in *P. lophurae* in ducklings (64) and chickens (95).

Phagocytosis of abnormal numbers of normal erythrocytes in malaria has been attributed to various immunological entities, both antigen and antibody, including an opsonizing antibody that is said to develop via an autoimmunity mechanism. However, George *et al.* (42) concluded in recent studies that red cell destruction in *P. berghei* infection was caused by hy-

TABLE 3. Transformation by *Ascaris* antigen of human lymphocytes from an *Ascaris*-sensitive person

ANTIGEN ADDED	DAYS OF CULTURE	SIZE OF CELLS (MICRONS)				TOTAL % OF CELLS 7 μ OR MORE	TOTAL % OF CELLS 9 μ OR OVER
		<7 μ	7 μ to <9 μ	9 μ to <11 μ	>11		
PHA	3	37 (±8.1)	30.25 (±5.2)	20.5 (±4.7)	12.25 (±5.4)	63 (±8.3)	32.75 (±8.2)
PHA	5	9.25 (±3.49)	24.25 (±6.38)	41.5 (±5.41)	25 (±10.17)	90.75 (±3.6)	66.5 (±6.9)
Ascaris 0.06 mg/ml	3	58.75 (±18.6)	29.5 (±6.1)	10.25 (±7.69)	1.5 (±0.87)	41.25 (±21.5)*	11.75 (±8.39)*
Ascaris 0.06 mg/ml	5	68.5 (±21.7)	24.25 (±4.6)	6.75 (±1.5)	0.5 (±0.5)	31.5 (±3.3)*	7.25 (±2.0)
Ascaris 0.006 mg/ml	5	83 (±3.81)	13.0 (±1.87)	3.75 (±1.92)	0.25 (±0.13)	17.00 (±3.75)	4.0 (±2)
Ascaris 0.006 mg/ml	3	80.5 (±25.49)	17.75 (±3.24)	1.75 (±1.3)	0	19.5 (±4)	1.75 (±1.29)
Control	3	87.75 (±1.47)	9.75 (±0.8)	2.5 (±1.12)	0	12.25 (±1.4)	2.5 (±1.12)
Control	5	81.75 (±26.04)	14 (±2.92)	4.25 (±2.58)	0	18.25 (±3.3)	4.25 (±4.08)

TABLE 4. Transformation by *Ascaris* antigen of rat lymphocytes from rats immune to *Ascaris suum*

ANTIGEN ADDED	DAYS OF CULTURE	SIZE OF CELLS (MICRONS)				TOTAL % OF CELLS >7 μ
		<7 μ	7 μ to <9 μ	9 μ to <11 μ	>11	
PHA	3	55.75 (±2.45)	35 (±5.2)	8.75 (±5.85)	0.5 (±.5)	44.25 (±9.29)
PHA	5	68.25 (±2)	15.75 (±3.3)	7.75 (±3.87)	8.25 (±3.15)	31.75 (±2)
Ascaris 0.06 mg/ml	3	91.5 (±1.4)	6.5 (±2)	2 (±1.23)	0	8.5 (±2.65)
Ascaris 0.06 mg/ml	5	88 (±1)	3.25 (±1.7)	6.0 (±1.35)	2.75 (±1.35)	12 (±1)*
Ascaris 0.006 mg/ml	3	96.25 (±10.9)	2.0 (±1.2)	1.0 (±1.2)	.25 (±.14)	3.25 (±.35)
Ascaris 0.006 mg/ml	5	93.25 (±1.68)	3.75 (±1.65)	1.75 (±2)	1.25 (±.26	6.75 (±1.78)
Control	3	97.25 (±1)	2.75 (±1)	0	0	2.75 (±1)
Control	5	96.5 (±1.4)	2.75 (±.7)	0.5 (±.9)	0.25 (±.14)	3.5 (±1.44)

Values represent percentage of cells in each group.
400 cells measured in duplicate cultures for each set.
Figures in parenthesis = standard deviation.
* Significant difference ($p = <0.01$) between test and controls.

persplenism and not by an opsonin. It was suggested that circulating parasites, even in low numbers, stimulate the phagocytic capacity of the RE system, which leads to an increased rate of sequestration of blood cells in the spleen, including normal ones, which ultimately produces a cumulative effect of greater splenic function and greater cell destruction. These authors were unable to detect IgG on red cells and presumed the absence of IgM because of the absence of agglutination and hemolysis. In a consideration of the possible mechanisms of the immunopathology of malaria, Dixon (35) has suggested a mechanism based on the formation of antigen-antibody complexes, unrelated to the erythrocytes, that nonspecifically adsorb to the surface of red cells with the subsequent fixation of complement. Such cells are then liable to lysis, which would explain intravascular hemolysis and erythrophagocytosis.

Erythrophagocytosis has been claimed as a cause of anemia in several other intracellular parasitoses—for example, *Babesia* in rodents (92) and *Toxoplasma* in humans (54).

Phagocytic activity is also seen in helminth infections, especially where tissue destruction occurs. In parasitosis of the lungs, for example, hemosiderin is frequently seen in macrophages, but apart from this there is little evidence of a direct action of macrophages on parasitic stages of parasites *in vivo*, though this is presumed in view of the frequency of macrophages in areas where parasites are or have migrated. In this case, it is likely that they are performing a purely phagocytic function without reference to, for example, delayed hypersensitivity mechanisms.

The adherence of peritoneal macrophages to the surface of second-stage *Ascaris* larvae when these are placed in the peritoneal cavity of mice immune to *Ascaris* has been reported (26), and this finding contrasts with the results of the author (97), who failed to observe a reaction with second-stage larvae of *Ascaris* in the peritoneal cavity of rabbits immune to *Ascaris*. It is possible, however, that different animal hosts respond differently.

Cytophilic antibody

Cytophilic antibody (*sensu stricto*) was originally described by Boyden (12) as a globulin component of serum that would become attached to certain cells so that the cells would then specifically absorb antigen. The original description was applied to a class of rabbit antibodies that would attach to spleen cells of rabbit or guinea pig. Another type of cytophilic antibody, apparently distinct from the spleen cell cytophilic antibody and capable of becoming attached to macrophages but not to most other cells, has now been described (13). The role of cytophilic antibodies in delayed hypersensitivity has recently been assessed by Nelson and Boyden (72), who conclude that thus far there is no clear-cut evidence of a definite role for cytophilic antibody in delayed hypersensitivity.

Preliminary studies on the possible occurrence of cytophilic antibody in *Ascaris* infection have been made in the author's laboratory. None has been found in the infection, but difficulty has been encountered in obtaining a satisfactory bis-diazotized benzidine linkage of *Ascaris* antigen to red cells, and radio-labeled antigen has not been investigated.

The possibility of an antibody cytophilic for lymphocytes has been suggested (72) as a result of work by Koprowski and Fernandes (57), who showed that normal lymphocytes, when treated with serum of rats immune to guinea pig spinal cord tissue, acquired an affinity for cultures of cells of the brain of puppies.

It would seem worthwhile, in view of the strong association of immunity to certain parasites with cells, especially lymphocytes and macrophages, to examine for the various cytophilic antibodies, especially where the spleen plays an important role in erythrophagocytosis.

Neutrophils

Neutrophils are the characteristic cells found early in parasitic infections, especially where there is inflammation and tissue destruction, and local foci of neutrophils are frequently seen around dying or dead parasites in various tis-

sues of the body. At times neutrophilia may be massive and may lead to an increase in the pathological changes associated with the infection (30). In experimental infection of the mouse with *Fasciola hepatica,* the later stage of migration of t' .s parasite in the liver is associated with a ıassive outpouring of leukocytes into the tra cs left by the migrating liver fluke.

The ear y neutrophil response is usually replaced b· one of lymphoid elements and macrophages and possibly this represents the onset of a spe.ific reaction to the parasite by sensitized cell.s. There is much to commend this idea, since second infections frequently produce an initial lesion beginning at the lymphoid stage rather than at the neutrophil stage.

The role of the neutrophils, like that of the other cells, in parasitic infections is unclear. They are part of the early response to a parasite and appear before well-formed immunologic reactions are in operation. They are also seen in certain immune reactions, particularly the Arthus reaction, but in both cases they are a response to inflammation and vascular injury with subsequent sticking of cells to the blood-vessel endothelium and the migration of the cells to the local tissue.

An account of the substances that promote leukocyte migration has been given by Hurley (50), who suggested that a factor (or more than one) of serum was activated by damaged tissue rather than by direct liberation from injured cells.

The role of antibody in the chemotaxis of polymorphonuclear leukocytes (PML's) was demonstrated by Boyden (11), who showed that the incubation of antigen-antibody precipitates in rabbit serum caused the serum to become markedly chemotactic to rabbit PML's. Studies with PML's and *Ascaris* antigens and antibodies have demonstrated a similar finding (25).

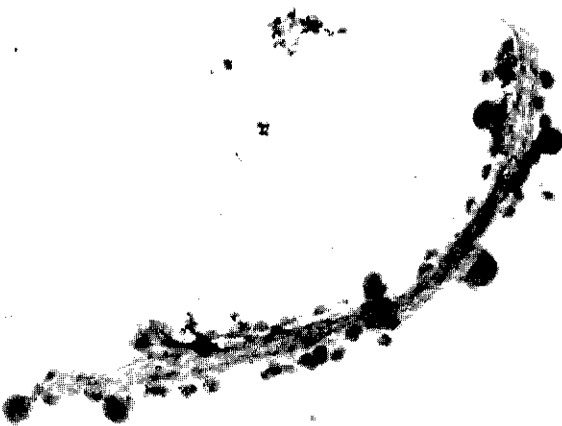
Adhesion of neutrophils to the surface of parasites

In vitro studies of the adhesion of neutrophils to the surface of second-stage larvae of *A. suum* have been in progress at the author's laboratory. When sensitized with antibody, second-stage larvae readily become covered with large numbers of neutrophils. Stained preparations of antibody-sensitized larvae exposed to buffy coat preparations of rabbit blood show the cells to be mainly PML's with a few adherent eosinophils. No pyroninophilia is seen with methyl green pyronine, and the cells do not show specific fluorescence when exposed to a goat anti-rabbit globulin conjugated with FITC. Larvae sensitized with rabbit serum will attract PML's from both immune rabbits and guinea pigs and also polymorphs from normal animals. The reaction is antibody- and complement-dependent and is produced with a variety of immune sera; these include those that will cause the adhesion of pyroninophils from peripheral blood culture to third-stage larvae and antisera to various fractions of the adult *Ascaris* worm and to unrelated parasites such as *Turbatrix aceti* and *Necator americanus.* Even normal sera of bovine, rat, and guinea pig will produce the reaction. While the several types of immune serum might be expected to contribute to the reaction, the ability of normal serum to induce it was somewhat unexpected. However, other studies have shown that second-stage larvae of *Ascaris,* unlike third-stage larvae, will nonspecifically become coated with a serum component, and this may have the ability to fix complement and cause the adhesion of polymorphs.

Second-stage larvae of *A. suum* show a definite sequence of cell adhesion with immune serum, especially when examined with antigen-stimulated peripheral blood cultures after one, two, three, four, and five days of culture. The adherent cells from one-day cultures consist only of neutrophils and eosinophils; as the cultivation time increases, the adherent cells consist of a mixture of neutrophils with increasing numbers of pyroninophils, and finally the majority of adherent cells are pyroninophils with only a few adherent polymorphs. The adherence of pyroninophils to second-stage larvae, as with third-stage, is mediated by immune serum. Consequently, the adherence of PML's to second-stage larvae is to a great extent nonspecific,

FIGURE 4. Polymorphonuclear leukocytes adherent to second-stage *Ascaris* larvae. Stained pyronine (x 750).

FIGURE 5. Polymorphonuclear leukocytes and pyroninophils adherent to second-stage *Ascaris* larvae (x 750).



in that normal serum and normal cells equally will take part in the reaction.

The adhesion of blood leukocytes to the surface of other parasites has been variously reported. Leukocytes have been observed to adhere to the surface of schistosomes after the infected host has been treated with an anti-schistosome drug (71), and microfilariae of *Loa loa,* after treatment of the infection with diethyl-carbarazine citrate, may accumulate in the capillaries of the liver, where they are attacked and enveloped by macrophages (16, 114). It has been suggested that the drug sensitized the microfilariae to attack cells of the reticulo-endothelial system.

Reactions between polymorphs and flukes derived from X-irradiated cercariae of *F. hepatica* have been described by Dawes (31). Flukes weakened by irradiation show adherent polymorphs on the surface, and these cells then penetrate the epicuticle of the fluke and pass into the core of the cuticle, causing disruption of the epicuticle. It has yet to be determined whether this reaction is mediated by an antibody mechanism, but there is every likelihood that some serum factor is concerned.

Other examples of the adhesion of leukocytes to parasites include the adhesion of microfilariae to human leukocytes in the presence of serum of an infected person (84), the adherence of *Trypanosoma lewisi* to leukocytes of immune rats (59), and the adherence of the pathogenic trypanosomes to leukocytes of guinea pigs and rabbits (60). This last reaction has been used to differentiate strains of trypanosomes. Eosinophils from patients with tropical eosinophilia have been shown to adhere to the filariform larvae of *Strongyloides* and *Necator* and occasionally to microfilariae (8). In this work, cell preparations from persons infected with hookworm or *Ascaris* were also reactive, but those from normal individuals were nonreactive. It is clear from these various reports that a variety of granulocytic cells can become adherent to the surface of a variety of parasites, including protozoa. It has not been established in all cases that antibody is concerned in the reactions, but it is likely that both antibody and complement are concerned in most of them.

The effect of granulocytic cells on living larvae has not been determined. There are no reports of degranulation of the cells on the surface of parasites, but such events have not been searched for, and, in all, a large unexplored field is available for study in this direction.

Eosinophils

The eosinophil is probably the cell that is most traditionally associated with parasitic infection, and yet, despite its characteristic appearance, there are no definitive statements available on its function or the factors that mediate its accumulation—sometimes in a spectacular manner—in the tissues. Usually, the eosinophil is not part of the early acute inflammatory response but occurs at a later stage of inflammation when round cells and plasma cells become evident. To some extent, its appearance is an indication of the age of an inflammatory lesion, and the frequent association of eosinophilia with allergic disorders, and especially parasitic infections, has led to the presumption that this cell type is associated with immunity processes.

Among the many theories that have been devised to account for the accumulation of eosinophils either in the blood or locally, three have received more attention than others. These are variations on a theme to do with histamine and can be stated (a) histamine attracts eosinophils, (b) eosinophils contain an antihistamine, or (c) eosinophils contain histamine (5). A more recent study of eosinophils and eosinophilia (61) has helped greatly to clarify the factors that induce eosinophilia. Evidence has been provided that it is due essentially to antigen-antibody complexes: antigen alone, antibody alone, or the by-products of an antigen-antibody reaction have little eosinophilotactic activity. If this work is confirmed, it would provide an explanation for the marked accumulation of eosinophils in parasitic infection, since in many cases the stimulating antigen or antigens (the parasite) can persist for some time in the body. Eosinophils have also been shown to have a phagocytic function, being capable of ingesting antigen-antibody precipitates (4, 61, 90), this is also associated with degranulation of the cells, and it may be a mechanism whereby the antigen-antibody aggregate is inactivated or degraded. A variety of enzymes have been identified from the granules of eosinophils, including cathepsin, β-glucuronidase, nucleases, phosphatases, and peroxidases (myeloperoxidase, verdoperoxidase), but their exact function is still in doubt. Some evidence of degranulation of eosinophils on the surface of *Ascaris* larvae has been noted by the author, but this effect has not been studied in detail or examined for its possible effects on the cuticle of larvae.

The idea that antigen-antibody aggregates provide the eosinophilotactic response is an attractive one; however, there are several reports that indicate the occurrence of eosinophilia in circumstances where the immune response plays a small part in the mechanism. Arean (6) has shown that the appearance of eosinophils at the site of injected *Ascaris* eggs was too rapid to be explained by an antigen-antibody reaction, and marked eosinophilia has been observed in a child with visceral larva migrans but who was agammaglobinemic and showed almost a complete absence of immune globulins in the serum (47). There are many reports, published over the last few decades, indicating that antigens from several parasites (including *Ascaris*) will induce eosinophilia after an initial injection (41, 106). Early work in this area was almost certainly with animals (e.g., dogs) that may have been naturally sensitized to ascarids, but even the more recent experiments should be intepreted with care, since animals are commonly parasitized with a low burden of "normal" parasites and these may be sufficient to sensitize an animal to a cross-reacting antigen.

In a study of eosinophilia to *Toxocara canis* infection in guinea pigs, Olsen and Schulz (81) showed that the onset and extent of eosinophilia were somewhat dose-dependent; guinea pigs receiving the largest dose of eggs (5,000) showed eosinophilia on the second day, while those receiving only 50 eggs developed it on the tenth day. Maximum eosinophilia was seen approximately two weeks after infection in all cases. Schultz-Dale tests with antigens from *Toxocara* eggs showed a dose dependency between the number of eggs given and the time when reactions could be elicited. Maximal eosinophilic responses at about two weeks after infection have also been observed in other nematode in-

fections, such as *Trichinella spiralis* (89) and *Dictyocaulus viviparus* in cattle (109).

An interesting aspect of the work by Olsen and Schulz was that though the eosinophilia persisted in infected animals for 28 days, the *Toxocara* larvae did not persist for more than 14 days, as judged by digestion techniques.

Support for the idea that eosinophils are antagonists of histamine is obtained from studies on the mast-cell, eosinophil, and histamine levels in rats infected with *N. braziliensis* (110). A marked eosinophilia that occurred from 12 days after infection onward was associated with a marked decrease in the number of tissue mast cells; this was interpreted to indicate that the disruption of mast cells, with the release of histamine, served to attract eosinophils whose function was to remove the excess histamine (110). However, an alternative explanation might be that mast-cell degranulation was induced by a mast-cell-sensitizing antibody and that eosinophils were attracted by the resulting antigen-antigen aggregates.

There is little doubt that the role of the eosinophil in parasitic infection requires much more detailed study.

Mast cells

Earlier studies of the cell response to initial and repeated infections of *N. braziliensis* in rats demonstrated that connective tissue mast cells (connective tissue basophils) fluctuated in numbers during such infection (102). This has been confirmed by Wells (110), who showed that mast-cell numbers in the rat intestine fell markedly about the fifteenth day of infection and later rose to levels much higher than those prior to infection. It is unlikely to be fortuitous that at the same time these events occur there is also a loss of adult worms (self-cure) of an initial *N. braziliensis* infection. The concurrent increased accumulation of eosinophils has been mentioned previously.

Degranulation of mast cells can be brought about by a number of agencies, including trauma, bacterial toxins, heat, cold, and ionizing radiation (104). An additional mechanism is degranulation by an antigen-antibody interaction, and it is now well established that the mast cell plays an important part in the anaphylactic syndrome (70, 105). A detailed consideration of the various aspects of this is given by Keller (55). Essentially, mast-cell degranulation may occur on contact with antigen after active sensitization and, according to the animal species, after passive immunization with serum. Mota has postulated a "mast-cell-sensitizing" antibody that is nonprécipitating, appears early after immunization, and may be present in the blood for only a short time (67, 68). The most satisfactory method of demonstrating it is homologous passive cutaneous anaphylaxis, and in the rat its production is much accentuated by the use of *Haemophilus pertussis* vaccine. Consequently, it closely resembles the "reagin" type of antibody (69).

The occurrence of an antibody similar to reagin has been reported to be closely associated with immunity to *N. braziliensis* in the rat (76, 78), the reagin being detected in some rats immediately after the acquisition of resistance and in all rats one week later. High levels of the reagin were stimulated only by infection with living, adult worms and not by vaccination with worm extracts. Rats immune to *N. braziliensis* undergo severe anaphylaxis on intravenous injection of antigen, and since the gut of the rat appears to be the major shock organ (111), it has been suggested that a local anaphylactic reaction in the gut may be responsible for the termination of an adult worm infection (103).

It has been shown recently that anaphylactic shock induced by an unrelated antigen-antibody system will enhance passive immunity conferred with antiserum. This produces a significant expulsion of worms compared to rats which were only passively sensitized (10). In this circumstance, it appears that the anaphylactic lesion has increased the passage of immunoglobulin to the lumen of the bowel.

It would be tempting to ascribe this sequence of events to a degranulation of tissue mast cells in the intestinal wall brought about by an antigen-antibody interaction, possibly with the

characteristics of a mast cell sensitizing antibody. Conclusive evidence for this, however, must await further study.

There is ample evidence to indicate that helminth antigens do have a profound effect on mast cells. A principle of *Ascaris* will degranulate rat peritoneal mast cells (104) and studies with mice infected with *T. spiralis* have shown that local (subcutaneous) degranulation of mast cells is dependent on prior sensitization with the parasites (14). In the latter studies maximal reactions were obtained one month after infection, using either metabolic or somatic antigens of *T. spiralis*. Sensitization could be passively transferred to normal mice, though in this case mast-cell injury was less than that observed in actively sensitized mice. Mast-cell degranulation in skin pouches of mice immunized against *Strongyloides ratti* has been described (43); in this case "excretory and secretory" antigens of infective larvae proved more effective than somatic antigens.

Various other reports have indicated an increase in the number of mast cells in the skin of mice infected with *S. mansoni*, *Hymenolepis nana*, and *Syphacia* and in the skin of patients with schistosomiasis and filariasis (37, 38). An increase in the number of bone marrow mast cells (basophils) and an increased release of these and eosinophils into the blood of guinea pigs after the injection of *Ascaris* body fluid has been reported (18). Comparable work using a highly purified polysaccharide of *Ascaris* muscle showed that intraperitoneal injections caused infiltrations of primitive hematopoetic elements of the erythrocytic and leukocytic series into the liver (80).

Globule leukocytes

These cells have received occasional attention over the last several years, having been noticed especially in parasitic infection of the gastrointestinal tract of ruminants. Their relationship to parasitism and in particular to the immunological process has hitherto been somewhat unclear, since they have been found in the abomasal and intestinal mucosae of both infected and normal animals. Recently, however, a clear relationship between gastrointestinal parasitism and the globule leukocyte has been reported, the cell being common in the mucosae of parasitized animals but infrequent in worm-free animals (112). An increase in globule leukocyte numbers has been observed in the intestinal mucosa of rats infected with *N. braziliensis;* this was marked on the twelfth day and was coincidental with the self-cure mechanism (112).

The nature and function of the globule leukocyte have yet to be fully clarified. Some have regarded it as comparable to the "Russell body" cell of the plasma cell series, since by light microscopy it appears very much the same as that cell (36). The Russell body cell has been shown to contain immunoglobulin, and in the work by Crandall *et al.* (27) such cells showed intense cytoplasmic fluorescence with anti-μ heavy chain reagents. A re-examination of the identity of mast cells and globule leukocytes by Jarrett *et al.* (52) showed that in rat, sheep, and bovine three related cell types occurred that could be differentiated on their staining reactions with toluidine blue and fluorescence with acridine orange. One form of mast cell was commonly found in the lamina propria of the intestine, gastric wall, and peribiliary area in *F. hepatica* infected livers. The ultrastructure of mast cells and globule leukocytes was reported to be similar, but different from that of the "Russell body" plasma cell; of special interest was the fact that mitotic activity could be detected in mast cells and globule leukocytes. The globule leukocyte was suggested as an end cell of a range of mast-cell types, derived from the type of mast cell found in the lamina propria of the digestive tract.

If globule leukocytes are in fact a form of mast cell, then a function for them could be envisaged in gastrointestinal parasitism. They could, as for example in *N. braziliensis* infection, accumulate in the mucosa and under appropriate stimulation release biologically active amines, which in turn could alter the permeability of the gut mucosa for larger macromolecules and possibly also for immunologically competent cells.

Summary

A wide range of cellular reactions are associated with parasitic infections. Some of these are probably nonspecific responses to tissue injury and inflammation, whereas others appear to be mediated by an immunological response. Since protozoan and, especially, metazoan parasites are not only complex antigenic entities but may also cause tissue destruction, it is to be expected that the cell response to them will be complex. Perhaps this complexity has been an unattractive prospect to workers in the past, but with an abundance of information now available on the morphology, ultrastructure, physiology, and functions of the wide range of cell types that can be found in parasitic infections, there is much less reason for the field to be neglected.

Moderator: The discussion of this topic will be initiated by Dr. Biro.

DISCUSSION

Carlos E. Biro

My comment must of necessity be general in nature, and I should like to keep it short.

To begin with, when the cells involved in the immune response are mentioned, the first confusion that strikes me personally concerns the cells actually involved in the immune response itself—in other words, in the preparation or production of antibody—and those that are called in after the antibody response has taken place, as effecters of damage. I think that this is a very important distinction to make in studying immunity to parasites and the cells involved in this kind of phenomenon. It is necessary to study on the one hand the initiation of the immune response, and on the other the phenomena that occur afterward.

To put it very simply, the first kind of cell involved in an immune response is probably a macrophage, a phagocytic cell. Most antigens are particulate or large matter, and these will be the first ones involved. By some mechanism that need not be discussed here, they seem to be in touch with or communicate with cells of another origin, lymphoid cells, and these lymphoid cells or their relatives differentiate to give plasma cells.

Along this line of differentiation, a series of events takes place. The first seems to be the production of IgM. The second seems to be the establishment of immunological memory, and possibly the last would be the establishment of IgG production. Again, I do not think it is important at the moment to discuss which starts first.

I have deliberately left IgA production and delayed hypersensitivity in a separate, not-too-clear position, because I do not think their position *is* clear. The only thing that, at least from my point of view, is interesting is the association between these two. There seems to be a reasonable and growing body of evidence linking the presence of IgA to delayed hypersensitivity. Anti-IgA antisera are able to block some *in vitro* models that are probably representations of delayed hypersensitivity. This, I think, would be important in studying parasite immunity.

Now, once all these cells have been involved in the production of antibody or an immune response, in another totally different stage—again, I do this for the sake of simplicity—there will be antigen-antibody complexes. I include in this term the interaction between whatever produces the delayed hypersensitivity and the corresponding antigen. As a result, we can have histamine release, changes in vascular permeability, polymorphonuclear cells attracted to the site of the lesion, and eosinophilia.

I start at this end because, not being a parasitologist interested in immunology but an immunologist interested in parasitology, I have to. I now ask myself, Where within this scheme do I fit immunity to parasites? I find that this is not as big a problem as I would have thought. I know that my colleagues in different specialties who deal with immunological problems would like to have a special immune response for each of them. The transplant people want one, the parasitologists want one, and why not? Yet I think that economy in a living being leads to the idea of the immune response as probably, if not one event, a series of linked events. In

this case the only question that need be asked is what the characteristics of the antigen are, so that we can know something of the characteristics of the antibody produced or the immune response produced to it. We find here the first division I must make.

I have been a little uneasy today hearing you talk simultaneously about monocellular, unicellular, intracellular parasites, and about very big things like helminths. I think the problems are totally different. We are not going to advance by lumping them; we have to separate them. In the case of the helminth, we have to think of a parasite that is outside the organism; as Dr. Remington said, the gut is really in the outer world. Here, at least, we have to consider a parasite that is big and lives part of its life outside the being it inhabits. The other thing we have to think about is a parasite with a very resistant surface. In this context, the production of antibody, a protein capable of weak interactions would seem to be very little protection against the parasite. One would expect the intervention of cells very particularly at this level. At the other extreme are the protozoa, which are necessarily intracellular parasites, and here again we have a problem. Under normal circumstances, immunoglobulins do not, or appear not to, penetrate in normal form into the cytoplasm of healthy living cells; therefore, once the parasite is admitted into an intracellular space, the only point at which there will be interaction between antibody and the parasite will be in its passage between one cell and another, or after the rupture of one cell and before the parasite is admitted to the next.

These two different approaches—one to the bigger parasite, on which a humoral factor will have little or no effect, and the other to the intracellular parasite, in which the moment of the antibody's action must be just the one when the parasite is outside the cell—are what I want to call to your attention.

On the negative side, one might add that the immune response was not made for either of these types of problems. I know this is teleology, and I apologize for putting it this way. But, to repeat, what I mean is this: In the case of the big parasites, humoral factors will have a very slim chance of helping to eliminate infection. In the case of the intracellular parasites, too, perhaps research should be directed toward something different from the immune response. There are other examples, such as viruses, in which nature apparently needed some other kind of solution because antibody production was not good enough.

If this sounds negative, I am sorry; it is not meant to. It is meant only to separate out problems so that we can tackle them one by one, instead of lumping them together in this hard-to-handle mixture which is the field of parasitology.

Moderator: I think Dr. Soulsby presented very interesting evidence that the attachment of pyroninophilic cells around the surface of larvae may play a role in the actual destruction of the parasite, and this is to my mind a very important point. We know that in delayed hypersensitivity tissues suffer a lesion on account of lymphoid sensitized cells, and why should not the tissues of the parasite suffer the same thing? We know that lymphocytes from people who have delayed hypersensitivity will produce lesions of target cells *in vitro*, and why not on the parasites?

I should also like to provoke a comment by Dr. Soulsby on the eosinophils. In the not very distant past, people have been very interested in the role played by the eosinophils in the various stages of the immunological sequence. We know that the antigen-antibody interaction attracts eosinophils. The eosinotactic factor has not been isolated as the chemotactic factor that produces the attraction of neutrophils and results from the interaction of antigen, antibody, and complement. But there is probably such a thing as an eosinotactic factor produced by antigen-antibody interaction, and we also know that eosinophilia is very characteristic of many parasitoses, particularly of helminthiasis.

I remember a paper written in 1963 in which Dr. Soulsby considered and speculated about Spier's point of view on the participation of

eosinophils. To my mind, the sequence of events suggested by Spier called for too many hypotheses: First, phagocytosis of the antigen by neutrophils. Second, phagocytosis of the necrotic neutrophils by macrophages. Third, lymphocyte macrophage cells (hemocytoblasts) undergoing DNA synthesis and dividing to form medium and small lymphocytes. Fourth, some of the mononuclear cells involved in the inflammatory reaction being injured by antigen, becoming swollen, and forming numerous vesicles. Fifth, attraction of eosinophils by the injured cells penetrating their plasma membranes and causing disruption of the cytoplasm. Sixth, macrophages engulfing cell fragments with adherent antigen as well as eosinophils associated with these fragments. Finally, microphages losing their phagocytic activity and undergoing transformation into antibody-producing cells.

I believe Dr. Soulsby will agree that this concept is based on a chain of unnecessary and unproved steps. It recalls the old dictum known as Occam's Razor: *Essentia non sunt multiplicanda praeter necessitatem* (One should not multiply the hypothesis beyond necessity). The only thing we really know is that the antigen-antibody complex, as well as certain macromolecules, including *Ascaris* keratin, are strongly eosinotactic, and we also know that *Ascaris* extracts are potent histamine liberators, and there are indications that eosinophils may play a role in the removal and neutralization of histamine. These are a good many assorted things that might be put together, perhaps, but I do not know how.

Like the attraction of lymphoid cells in delayed hypersensitivity, the attraction of eosinophils under the conditions of Dr. Soulsby's experiments a few years ago still requires explanation, I think.

Soulsby: I agree entirely that Spier's sequence of requirements for his theory of antibody initiation and production is so complicated and requires so many unproved steps that it is more or less unacceptable. I think that nowadays almost everyone would agree.

If there was anything that would have made me refuse to cooperate in this conference, it was any consideration of the eosinophil. It is extremely difficult to find definite information about it. As you well know, there are so many theories about it—that it is a histamine carrier, that it is an antihistamine carrier. The recent publication of Litt's in the *Annals* of the New York Academy of Sciences seems to offer a fairly good explanation of eosinophilia in parasitic infections. He suggests that eosinophilia is very common in helminth infections because the antigen persists for some time in the tissues. Consequently, a residuum of antigen-antibody aggregates might be expected to persist there.

We have been able, using the *in vitro* technique mentioned earlier with larvae, to demonstrate the adherence of eosinophils to larvae. This is similar to Bang's demonstration of a comparable reaction with eosinophils and microfilariae in cases of tropical eosinophilia in India. The reaction depends on sensitization of the larvae by antibody and is also complement-dependent. This would fit with Litt's work.

I want to return for just a minute to the pyroninophil-adherence reaction. I want to emphasize that this system we are working with is a very artificial one. I would be the last to suggest that this is a definite *in vivo* mechanism. A point I raised in my presentation was that perhaps we should think not only in terms of the parasite, but also in terms of the local environment of the parasite. In examining a lesion, it may be seen that these cells are very numerous in the local environment. When the parasite is transferred to an artificial system, there is no longer any local environment, but in this situation cells are observed adhering directly to the parasite.

Moderator: I appreciate the caution of your statement.

Nussenzweig: I have two questions for Dr. Soulsby. What is the role of the complement in these reactions, and their relation to the immune-adherence phenomenon? Are the large cells already present in the lymph node of the animals or are they transformed on the surface of the larvae?

Soulsby: We produce these cells in culture

and then use them. It is only when cells have undergone transformation that they are active in the reaction. Cells will transform if they are cultured with living larvae, presumably as a result of the release of antigen into the medium. But we have never observed an adherence of unstimulated lymphocytes to larvae, and consequently it does not appear that they are stimulated to transform on the surface of larvae.

Goodman: I have a comment and then a question. The comment is the same one I made about immunoglobulins: that we do not really know the role of delayed hypersensitivity or of circulating antibodies in many of the classical infections by viruses and bacteria. Therefore, we should not expect to understand clearly the role of delayed hypersensitivity in parasitic diseases without a good deal of work. The gaps in our knowledge about the function of different kinds of circulating antibodies persist although we can purify them and even determine their structure. In the case of the delayed hypersensitivity system, on the other hand, we have very few *in vitro* techniques. It is good to hear that Dr. Soulsby is making a start on applying them to the parasitic diseases.

My question concerns the reaction he is working with; I believe it involves the lymphocyte, which has changed, been "transformed," become a blast cell. It has been very interesting to follow the development of *in vitro* systems devised to study delayed hypersensitivity; to note that Dr. Perlman in Stockholm and Dr. Brunner in Lausanne have been able to take sensitized lymphocytes (which have not previously been transformed) directly from the spleen (Brunner) or peripheral blood cells (Perlman) and mix them with the target cells (to which the animal has delayed hypersensitivity). The damage to the target cell can be determined by culturing them or by measuring the release of labeled chromium from the cell. Have your parasites been damaged by the transformed lymphocytes? Have you been able to apply the Perlman or the Brunner technique and expose any stage of the parasite to lymphocytes (either from peripheral blood or from lymph nodes or spleen) and show that these parasites then do not grow normally or cannot be transferred and grown normally when put into another animal? It would seem that parasites should offer an excellent system for this type of *in vitro* investigation of delayed hypersensitivity.

Soulsby: You have summarized our research for the next two years. It would be most interesting to be able to demonstrate a lesion on the parasite with these cells, but at present we have not detected any. But it may be that such a lesion takes longer to form than our system will demonstrate. The study of an individual preparation is terminated after about half an hour. We are, however, initiating work on the continuous *in vitro* culture of cells with larvae, and at present we have a little evidence that a parasite population survives less readily in a population of immune cells than of normal cells.

Biro: I still insist on my separation. Histamine release, polymorphonuclear attraction, circulating antibody, and immediate hypersensitivity all belong on one side, whereas delayed hypersensitivity—or, at least, so there is an increasing body of evidence to show—probably belongs on the other. I like to think of delayed hypersensitivity as the local manifestation of a secondary immune response. This is very important in relation to Dr. Soulsby's model, because he is dealing with the adherence of cells, and I think that the stickiness of the surface of a cell is what decides whether a cell will or will not circulate or recirculate. Not much immune response goes on in circulating cells. It is usually in cells that are stuck. What makes them stick? This is an important question.

In the adherence of blastoid or transformed cells to larvae, two different models, the meaning of which would be entirely different, have to be separated. One of them is as Dr. Soulsby has been carrying it out: the cells have already acquired this stickiness, which I tend to feel might be very nonspecific. A question I might ask is what happens when one takes another kind of barrier for immunoglobulin, another big particulate kind of antigen, and covers

it with its antibody. Does this also stick to the cells and not change the specificity of the antibody or of the cells but change the specificity of the carrier?

The other model is the one Dr. Goodman suggested, in which transformation would take place locally, on the cell. I think that this acquisition of stickiness shows the beginning of an immune response. It might be worth while to remember that these blast cells or blastoid cells do not appear in the lymph that comes from the site of antigen into a lymph node. They appear in the node and in the efferent lymph—the lymph going away from the node. It seems to take some kind of adhesion or mutual interaction between cells to get the phenomenon started. This is very difficult to correlate with *in vitro* models.

R. S. Nussenzweig:* I should like, if I may, to raise one more problem concerning the cellular aspects of host response to parasitic infections, in addition to those that have already been discussed: the stimulation of the reticuloendothelial (RE) system that is known to occur in many cellular and blood parasitic infections, the importance of which has been recognized since the classical work of Taliaferro. But I have the feeling that since then this problem has not had enough experimental attention and that many related problems remain to be clarified.

I want to present some data we obtained in malaria infection, using rodent malaria parasites in mice. Immunizing animals with sporozoites, the insect form of the malaria parasite, incorporated in Freund's complete adjuvant, we observed the development of a certain degree of protective immunity, shown by a two- to threefold increase in the survival time of the immunized animals. But similar results were obtained in a control group injected repeatedly with Freund's adjuvant alone, without parasite antigens.

We thought of RE stimulation as enhancing the natural resistance to the malaria infection, and the next step was to try out other—and if possible more potent—stimulants of the RE system to verify whether these effects could be reproduced. For this we used *Corynebacterium parvum*, which had been shown by Dr. Biozzi and his colleagues to have a very potent accelerating effect on carbon particle clearance. We treated animals (A/J mice) with killed *C. parvum* and later challenged them, and also untreated controls, with viable sporozoites of *Plasmodium berghei.* A quite extensive protection was observed: only 30 to 50 per cent of the pretreated animals became infected, compared to 100 per cent of the control animals. Furthermore, some of the pretreated animals that became patent survived the *P. berghei* infection; this has never been observed with this parasite under normal conditions. In conclusion, there was a marked increase in host resistance produced by this nonspecific stimulation of the RE system. I think it would be worth while to see whether something similar could not be observed in other systems with different malaria parasites, and even in other parasitoses.

Moderator: This is a very interesting observation. As far as I know, Dr. Biozzi never had a conspicuous degree of protection against bacteria with this stimulation. Against *Salmonella typhimurium* in the mouse, I believe he never got any protection.

R. S. Nussenzweig: I am not aware of published results on the effect of administering *C. parvum* on *Salmonella typhimurium* or any other bacterial infection. As for the effect of *C. parvum* in other systems, Halpern and his colleagues obtained a certain inhibition of tumor development and increased survival rates in several experimental tumors in mice pretreated with *C. parvum.* Biozzi and his colleagues also reported on a reduction of mortality rates due to graft-versus-host reactions in mice pretreated with *C. parvum.*

Dubos: Since the time is so short, I shall limit my remarks to Dr. Biro's category of biochemical events, taking place as a result of antigen-antibody reaction and also of allergic reaction, that reveal changes in the activi-

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ties of the cells due to the immunological process. He denoted the biochemical change by the word "histamine." He used histamine because this happens to be a substance that immunologists are professionally interested in, but I am sure that he meant it to symbolize many other kinds of biochemical changes that take place. It is a fact, as you well know, that during the past five or six years an enormous amount of information has been accumulating on the changes that occur in the physiological and biochemical activities of various types of cells in various immunological situations.

Let me just mention two or three that I believe are directly relevant to the question that has been worrying you today; that is, how the immune reaction exerts its anti-infectious effect on the pathogen, whatever the nature of the pathogen.

There is no doubt that after any one of the classical immunologic reactions there is a change in the metabolic activity of the cell concerned. This has been illustrated by the phenomenon so well known since Metchnikoff, and so well documented since, that a change in the glycolytic activity of the cell takes place, so that suddenly there is the classical burst of acidity that results from an increasing glycolytic activity associated with phagocytic processes, for example.

Moreover, and also extending early observations of Metchnikoff's, there has been spectacular demonstration that not only as a result of phagocytosis but also as a result of mere contact between the antigen and the immunologically competent cell, explosive changes occur in the different types of granules of the cells—to such an extent that the phenomenon has been described as degranulation. This might more properly be referred to as the destruction or explosion of the lysosome, with a sudden release into the cell and the vicinity of the cell of all the components of the lysosome system, which in the final analysis means all the enzymes that are stored in the lysosome complex.

There is also a great deal of evidence that almost any kind of stimulation of an active cell by the antigen to which it has been sensitized results in an accumulation within that cell of many types of biochemical equipment, as can so well be demonstrated by growing macrophages in tissue cultures and showing that the richness in different metabolic potentialities can be changed at will.

It seems to me that you should have all this in mind. You will find that the question you have been asking all day—how does the immune mechanism really affect the susceptible cell?—is probably much simpler than we knew a very few years ago.

By the way, let me say that your questions are not at all peculiar to parasites; they apply to all bacterial species. They apply exactly to immunity against tuberculosis. I amused myself by taking several sentences from Dr. Smithers' presentation and inserting "tubercle bacilli," and I found that his text was absolutely applicable, with the same queries, the same uncertainties, but also I believe the same possibilities of explanation.

So let me just suggest that in your thinking about this problem you bear in mind that the activities of the cells involved in the immune process are profoundly affected by any form of immunologic reaction, whether it be the classical antigen-antibody or the delayed allergic type of reaction. I believe you will find then that most of the phenomena of protection that appear so mysterious are due in reality not to a direct effect of the antibody on the parasite (whether virus, bacterium, or helminth) but to the secondary manifestations of the allergic process—the release of a whole variety of chemically metabolically active substances all produced in Dr. Biro's second category.

Moderator: I think we had better proceed now with Dr. Weinstein's presentation. I hope we may take up this matter again later.

IMMUNOLOGIC ASPECTS OF PARASITIC INFECTIONS

Paul P. Weinstein

The effects of the immune response on parasites are manifested in a wide variety of ways, and many studies have been devoted to a description of diverse aspects of the response, such as behavioral and morphologic changes, structural damages, changes in infectivity and in subsequent growth and differentiation, and metabolic alterations. In few instances, however, has there been a systematic attempt to study in detail and interrelate the variety of consequences to a parasite that may follow an immunologic event.

For the sake of discussion, the effect of the immune response on parasites will be arbitrarily divided into several levels of reaction: gross, physiological, and biochemical. It is quite evident, however, that these categories are artificial and may operate simultaneously in a complex interplay. Therefore, the description of the consequences of an immunologic reaction upon a parasite will, like the proverbial blind men and the elephant, depend upon the analytical tools that are brought to bear and upon the perceptiveness of the investigator.

No attempt will be made here to present a detailed review for each of the subjects discussed. Instead, selected examples will be used in an attempt to assess the major reactions exhibited by protozoa and helminths. Many of the observations that will be referred to have been made *in vitro* only, and their relevance to the *in vivo* condition is frequently not clearly understood. It is similarly apparent that in most instances insufficient information is available for determining whether a given reaction plays a role in the ultimate destruction of a parasite. It is unfortunately still true that the underlying mechanisms of protective immunity in the great majority of parasitic infections are a mystery.

Gross aspects of reaction

Immobilization reactions

Antibody acting on various species of protozoa and helminths may result in retardation in movement and immobilization.

Entamoebae. This type of reaction commonly exhibited by amoebae was first described by Cole and Kent (8) for *Entamoeba histolytica* exposed to the serum of rabbits that had been immunized to the organism. When placed in antiserum, motile trophozoites soon ceased to form pseudopodia and rounded up. Maximal immobilization was obtained in twenty to thirty minutes, after which the amoebae regained their activity. Serum from infected humans was also shown to possess the immobilizing factor (3). Previous investigators had considered that surface antigens were involved, and Biagi *et al.* (2) provided data to strengthen this hypothesis. Using fluorescent-antibody procedures, they observed that as immobilization occurred, the fluorescence was localized principally on the surface of the organism. After 45 minutes, it was regularly distributed in the ectoplasm and endoplasm. At 60 minutes, activity in the cytoplasm was noted with the appearance of non-fluorescent areas. At 75 minutes, all the fluorescent material was

present in a large vacuole in the endoplasm, and at 105 minutes it was barely perceptible; at this time remobilization of the trophozoite was observed. Loss of fluorescence was not seen in trophozoites that were not remobilized.

Rabbit antiserum to *E. histolytica* also markedly inhibited the ability of the amoebae to ingest red blood cells *in vitro*, apparently because of the immobilizing effect of the antiserum (63).

Similar immobilization reactions have been shown to occur with *E. invadens*, *E. coli*, *E. ranarum*, and *E. moshkovskii* (89). The first two species with *E. histolytica* fall into one antigenic group, distinct from the two latter species. Biagi *et al.* (2) believe that the antibody responsible for the immobilization reaction is a gamma globulin. Antiserum that is not inactivated will produce lysis of the amoebae; the inactivated antiserum produces, in addition to immobilization, a certain degree of agglutination (89).

According to Zaman (89), a strain of *E. invadens* when grown in the presence of specific immobilizing antiserum appeared to change its antigenic structure; growth in the presence of antiserum was very poor. However, when transferred back to medium with normal serum, it reverted to the original antigenic type.

Trypanosoma cruzi. Immobilization and structural changes of *T. cruzi* have been noted *in vitro* by Adler (1). Specific serum added to normal cultures results in rapid agglutination, chain formation, and immobilization of the large majority, though not all, of the flagellates. Flagellates exposed to concentrations inhibiting growth lose their capacity to multiply several days before flagellar activity ceases completely. Specific serum results in apparent fusion of some flagellates owing to the destruction of the cell membranes of agglutinated individuals. The trypanosome form appears to be less susceptible to the immobilization effect of specific serum than the crithidial. Lysis of the crithidial form of *T. cruzi*, and in some cases of the trypanosome stage, by normal sera from various animals frequently occurs. Warren and Borsos (88) have shown by immunologic methods that fowl sera contain at least two factors against the crithidial form; a heat-stable factor, which is in all probability a strongly agglutinating and sensitizing antibody, and a heat-labile factor, which is probably complement. It was concluded that the agglutinating factor (antibody) is due to response to a cross-reacting antigen possibly present in a common contaminant of fowls.

Balantidium coli. When added to diluted fresh serum from immunized rabbits, the ciliates were rapidly immobilized and lysed. However, inactivated antiserum produced immobilization almost immediately, without lysis. Although the organisms remained stationary during the reaction, local ciliary movement continued with gradually diminishing activity; after 6 to 7 hours disintegration occurred. Inactivated normal rabbit serum had no effect on the activity of *Balantidium* (90).

Miricidial immobilization. Immune serum capable of rapidly immobilizing miricidia of *Schistosoma mansoni* has been reported for infections with *S. mansoni* (61, 29), *S. japonicum* (61), and *S. douthitti* (29). The reaction occurs in sera inactivated at 56° C for 30 minutes and is due to the clumping and immobilization of the surface cilia. In the higher dilutions of immune serum, some of the cilia on a given miracidium were clumped while others on the same organism continued to beat normally. The miracidia remained alive, as determined by flame cell motion, which apparently was unimpaired during the period of observation (62). No immobilization occurred in sera from uninfected control animals. The evidence was that the response was due to an antigen-antibody reaction: activity was completely abolished by absorbing serum with schistosome eggs, adults, or cercariae, and lyophilized preparations of these worm stages injected into animals gave rise to immobilizing antibody. Miracidial-immobilization factors were also demonstrated in all the globulin fractions of a schistosome patient's serum (34).

Using fluorescent-antibody reaction, Sadun *et al.* (57) reported that the ciliated epithelium and cilia of the miracidium stained brightly in immune serum, whereas the remainder of the organism was nonreactive.

Electron microscopy has revealed alterations in the surface of miracidia of S. *japonicum* exposed to serum from infected rabbits (28). The epithelial cells were greatly swollen, and their outer surface was covered with a protein complex that appeared as a coat of amorphous material. The most striking change was the swelling of the cilia, which caused their axial filaments to bend and coil. No alteration of subepithelial structures was observed. The miracidia incubated in normal serum showed no change in surface or internal structure.

For *Fasciola hepatica* infection in sheep, the findings are somewhat different (66). Heat-inactivated sera from either infected or normal animals have no miracidial-immobilizing activity. However, immobilizing and cytotoxic activity is quite marked in both types of sera in the presence of complement, with some slight indication that specific augmentation of the reaction may occur during the early stage of infection; it is at such a period that antibodies to *F. hepatica* infection are highest.

Membrane and Precipitate Formation

Trematodes. The cercarienhüllen reaktion (CHR), also referred to as the cercarial-envelope reaction and pericercarial-membrane phenomenon, was first described by Vogel and Minning (85, 86) and has subsequently been studied by several other investigators. Vogel and Minning noted that cercariae of S. *japonicum* and S. *mansoni* incubated in serum from schistosome-infected animals soon became invested with a transparent membrane. Detailed observations made by Stirewalt and Evans (71) using phase microscopy revealed that the cuticle of cercariae of S. *mansoni* in serum from uninfected mice appears as two very dark green to black parallel lines separated by a clear yellow-orange to orange-green space about 2 to 3 microns wide. The cercariae remained actively motile and showed no cuticular changes for several hours. In serum from infected mice, however, changes occurred rapidly. Within a few minutes, large amounts of a sticky material were secreted orally. Soon thereafter the width of the cuticle increased, as though the cuticular material was swelling or fluid was collecting within it. Associated with this was a shift in its color from orange to a deep green. These changes spread over the entire cuticle during the course of the first hour and were followed by the apparent detachment of the swollen area, which resulted in the ensheathment of the cercariae in a transparent membrane. The membrane was pliable at first but became cross-ridged in consequence of the cercarial movement and soon "set" and hardened. Finally, it loosened and ballooned away, encasing the cercaria as though it was in a mold. Of particular interest is the finding that schistosomules that were rapidly recovered following penetration of the host did not form the CHR reaction (70). This suggests that a surface change occurred in the parasites with their penetration of skin.

The CHR serum factor is relatively thermostable, though strongly reacting serum when heated at 56° C for 30 minutes showed a marked reduction in its capacity to induce envelope formation. The addition of unheated normal guinea-pig serum not only restored the CHR factor, but enhanced it. The rapid formation of the pericercarial envelop apparently protected the organism against any "cidal" activity of the serum (71). Serum fractionation studies led to the suggestion that the gamma globulin in infected mice contains both the pericercarial envelope-forming and cercaricidal factors. Inhibition of the cercaricidal factor appeared to depend upon the presence of an alpha globulin component perhaps aided by dilution with albumin; such inhibition allowed the expression of the CHR factor present in the gamma globulin (20). Further investigation with serum from humans infected with S. *mansoni* demonstrated that both the CHR and cercarial agglutinating activity were present in an electrophoretically homogeneous, fast-mov-

ing portion of the gamma-1-globulin (T-globulin). Ultracentrifugal analysis showed the fraction to be polydisperse, having two minor comparatively fast-sedimenting components (S_{20}=11.9 and 7.7, respectively) and a major, slowly sedimenting component (S_{20}=5.1). CHR and cercarial agglutinating activity were isolated in the major component (19).

Nematodes. Precipitate formation at one or more of the various apertures of nematodes (excretory, oral, anal, reproductive) and within the lumen of the gut has been described for many species of nematodes incubated in serum from infected animals. Sarles and Taliaferro (59) and Taliaferro and Sarles (79) first described this reaction as occurring *in vivo* in the tissues of rats showing a marked immune response to *Nippostrongylus braziliensis*. A detailed description of the reaction developing *in vitro* against various stages of the living parasite was presented by Sarles (58), who demonstrated that it would occur in heat-inactivated serum. The addition of complement had no further effect and did not elicit lytic or lethal reactions. Since then the formation of similar precipitates on various other species has been demonstrated: *Trichinella spiralis* (47), *Ascaridia galli* (55), *Heterakis spumosa* (64), and *Oesophagostomum radiatum* (52).

Although there is a possibility that precipitate formation is correlated in some instances with decreased activity of the worm (58), the results have been inconclusive or negative for the most part. In a study with *Neoaplectana glaseri* (27), the organisms grew to the adult stage in culture in the presence of specific antiserum from immunized rabbits. No change in activity or injurious effect was noted, despite the development of precipitates at all the various openings through which excretions and secretions passed. In a comparable study *in vitro* with *Oesophagostomum radiatum*, Douvres (52) found no evidence that the antibodies responsible for precipitate formation adversely influenced the development of the larvae. In this latter study it was also reported that a "coating" of the body surface of larvae occurred, and that both the precipitate and the coating phenomena took place only in intestinal tissue extracts from infected or immune animals, not in sera from these hosts.

Evidence that the precipitates forming on nematodes are antigen-antibody complexes was presented by Jackson (25, 26), who demonstrated that fluorescent antibody was specifically incorporated into the precipitates developed at the orifices of *N. braziliensis* and *Trichinella spiralis*. Similar fluorescent antibody findings have been reported for *Ascaris lumbricoides* (75) and *A. suum* (75, 9).

Physiological aspects of reaction

The immune response to helminths is frequently manifested by a repression of growth and differentiation that results in various degrees of retarded development and, with adult worms, in inhibition of egg production. Michel (39), in his analysis of these phenomena exhibited by various species of nematodes parasitizing domestic animals, has suggested that inhibition of development should be distinguished from stunting or interference with growth. True inhibition of development arrests the growth of the worm at a precise point in its life cycle (either third, fourth, or fifth stage), which varies among species. For example, the great majority of *Ostertagia ostertagi* become inhibited early in the fourth stage and the resulting worm population is very uniform in size. The worms may survive in the tissues of the host for a relatively long period. They may ultimately resume their development and become undersized adults, these now being considered "stunted" organisms (39). If this differentiation between inhibition of development and stunting proves to be generally correct, it would imply an effect of the immune response, directly or indirectly, on the growth-regulatory mechanisms of nematodes. That such inhibition of larval development is based on immune reaction has been deduced from several lines of evidence. Inhibition is readily reversed upon the transplantation of larvae from the immune to a nonimmune

host, as has been shown for *N. braziliensis* (59, 7), *Oesophagostomum radiatum* (52), and *Haemonchus placei* (51); the transplanted organisms rapidly develop to mature adults. Soulsby and Owen (68), using an alkylating agent, chlorambucil, were able to depress the immunity of sheep containing a large population of inhibited nematode larvae. The progressive fall in hemagglutination and complement-fixation titers and white blood cell counts was inversely related to the steep rise in fecal egg counts. Similarly, as is discussed by Soulsby (67), a reactivation of dormant larvae due to a decrease in the level of immunity during the winter months may contribute substantially to the so-called "spring rise" in parasitism seen in domestic animals. It should be pointed out, however, that serologic response and the existence of protective immunity frequently show little correlation in parasitic infections.

Reduction in the infectivity of larvae after relatively brief treatment *in vitro* with immune serum before being introduced into a host may represent another facet of larval inhibition. Observations on various nematode species have been reported: *Ancylostoma caninum* (48), *T. spiralis* (37), *N. braziliensis* (81). This effect was considered to be due to antibody, since the sera reducing infectivity also caused precipitate formation at larval orifices and in the case of *T. spiralis* were demonstrated to contain complement-fixing antibody and immunizing capacity on passive transfer. This effect in the case of *T. spiralis* was associated with the euglobulin fraction of the serum (38).

The immune response to nematode infection is also frequently characterized by a decrease in egg production, as for example with *N. braziliensis* (59, 42) and *Ostertagia ostertagi* (39). Inhibition of ovulation, although it superficially appears to result from the stunting of the worms, seems on analysis to be a separate phenomenon. This conclusion is based on the fact that within any worm population in an immune host there was no correlation between the size of a female and the number of eggs it contained (39). Evidence that inhibition of egg-laying is a reversible phenomenon was presented by Chandler (7) and Mulligan *et al.* (42). When adult *N. braziliensis* females showing inhibited egg production in immune rats were transplanted to nonimmune animals, their egg-laying capacity was rapidly restored.

It appears highly probable that the various phenomena just described are due primarily to an immune response. However, it is not at all certain that antibody acting directly on the organism constitutes the complete explanation. For example, at a particular period in an intestinal nematode infection, the organism may be expelled from the gut, which will result in a fairly abrupt diminution of the infection. This reaction, first observed by Stoll (72) in haemonchosis in sheep in response to challenge with infective larvae, has its counterpart in *N. braziliensis* in the rat, in which the original infection rapidly induces a sufficiently intense immune state to result in expulsion of the worms. Stewart (69) demonstrated that a hypersensitivity reaction of the immediate type, resulting in edematous and histological changes in the gut mucous membranes, occurred in association with "self-cure," and discussed the possibility that such environmental alterations in worm habitat might be involved in the mechanism of the reaction. In studies on the mechanism of the self-cure reaction in *N. braziliensis*, it was similarly postulated that the importance of antibody lies not in its direct action on the parasites, but in its inducing a state of hypersensitivity in the gut, in which fixed antibody and worm antigen would give rise to a local anaphylactic reaction. Such a reaction could conceivably render the parasite's environment "unsuitable" (42, 84). The same response, however, could be induced by the passive transfer of serum, and it was recognized that a direct action on antibody on the worms in the intestine need not necessarily be excluded by this interpretation. Increased capillary permeability associated with local anaphylaxis could well result in a leakage of

plasma proteins containing antibody, and in fact it was found that immune serum did significantly inhibit worm oxygen uptake after overnight incubation (42).

The same concept, but based on a different immunological mechanism, has been invoked for *T. spiralis* infection. Larsh (33) has concluded that the mechanism causing the expulsion of the adult worms from the host by creating an unsuitable biochemical environment is triggered by a specific delayed, rather than immediate, hypersensitivity reaction in the gut. Gordon (22) has objected to this interpretation on the ground that the protection-giving cell types used were not lymphocytes and that other pertinent controls were not included. Although Kim (31) has reported the development of delayed hypersensitivity to *T. spiralis* antigen, he failed to accomplish passive transfer of immunity by splenic cells. Wagland and Dineen (87), have presented more convincing evidence for the cellular transfer of immunity to *Trichostrongylus colubriformis* in an isogenic strain of guinea pig, using mesenteric lymph nodes as their cell source. But since they did not employ normal cell controls, the results are difficult to evaluate. Findings suggestive of transfer of immunity to *A. caninum* in pups by serum and lymphoid cells have also been reported recently (40). It is obvious from these various findings that much work remains to be done in this interesting area of research.

If the idea of anaphylactic shock inducing the expulsion of nematodes from the gut is a valid one, then it seems from the results of Stewart (69) and Ogilvie (45) with *N. braziliensis* that the reaction must be "worm"- but not necessarily species-specific. A severe anaphylactic reaction induced by an unrelated antigen-antibody reaction (egg white) had no detectable effect on the worm population in the shocked rats. Furthermore, when homologous shock was induced in rats by the injection of worm antigen 28 days after the initial infection, at which time most of the worms had already been expelled, the small residual population was found to be unaffected. Ogilvie (45) points out that this result does not necessarily mean that a local worm-specific anaphylactic reaction fails to remove the major part of the worm population; worms that survive may do so because of their ability to endure such a reaction.

Ogilvie (44, 45) has presented evidence that antibodies resembling human reagins are closely associated with both the primary and the secondary immune response of rats to *N. braziliensis* infection, and that stimulation of reagin production is related to infection with living worms. She has suggested that these antibodies may be involved in protective immunity, but no mechanism has as yet been proposed. The effects of reagins in protecting against *S. mansoni* infection have been equivocal (46), and the results against *S. japonicum* have been negative (24).

Although for many years the matter of protective immunity occurring in schistosomiasis was in considerable doubt, the evidence that has gradually accumulated from epidemiological and laboratory studies, particularly during the past two decades, indicates that it does develop at least to some degree in various host species (30, for review). The immunologic mechanism involved is still obscure. The stunting of worms and diminished egg excretion have been reported to occur in the case of *S. mansoni* in the rhesus monkey (43) and in the rat (65). The latter authors, however, have rightly pointed out that the stunting could be due to a failure of these abnormal hosts to supply an essential nutrient or provide some other physiological need of the worm. However, in the case of *S. japonicum* the stunting of worms is perhaps more likely to be due to an immune response (56).

Biochemical aspects of reaction

One of the most interesting areas of immunological research concerns the effects of antibody on cell metabolism and physico-chemical structure, yet relatively little work has been done in this field with parasites. The first such study was performed with *Trypanosoma lewisi,*

a parasite of the rat. The parasites reproduce actively in the blood of the host during the first few days of an infection until a crisis occurs in which trypanocidal antibody destroys most of the organisms. Reproductive activity also diminishes during this period, so that shortly before or after the crisis there is a complete cessation of division. The survivors remain in the blood for several weeks or months as nonreproducing adults. Taliaferro (76) demonstrated that this latter phenomenon is brought about by the development in the rat of a passively transferable serum component that specifically inhibits the mitosis and growth of the parasites without detectably affecting their vitality or infectivity. This serum component was named ablastin (77); unlike the trypanocidal antibodies, it does not sensitize trypanosomes *in vitro* and cannot be removed from immune serum by absorption with living parasites (77). In a study of the biochemical changes that occur during the course of the infection, Moulder (41) demonstrated that trypanosomes inhibited by ablastin have a higher oxygen uptake and higher respiratory quotient, but a lower glucose utilization, than the dividing forms. On the basis of effects with metabolic inhibitors such as azide and dinitrophenol, he suggested that ablastin may inhibit the oxidative assimilation of glucose in reproducing trypanosomes in such a manner as to stop cell division and growth. In further studies using isotopically labeled amino acids and adenine, Taliaferro and Pizzi (78) demonstrated that inhibition of the division and growth of *T. lewisi* in the presence of ablastin was accompanied by an essentially complete cessation of nucleic acid synthesis, and inhibition of protein synthesis by more than 50 per cent. Ablastin also markedly inhibited the uptake of certain purines, pyrimidines, and nucleosides (50). Using *in vitro* studies, D'Alesandro (11) found that ablastic serum inactivated at 56° C for 20 minutes did not lose its inhibitory activity, which indicates that ablastin is not complement-dependent. Parasites grown at room temperatures were not affected by the antibody, which suggests that there are basic antigenic differences between bloodstream forms at 37° C and culture forms at room temperature. Ultracentrifugal studies (10), revealed that both ablastin and the early trypanocidal antibody are associated with a globulin component of a small molecular weight with a sedimentation constant of 6S. D'Alesandro (11) has discussed possible modes of action of ablastin but was unable to reach any specific conclusion. Lactic dehydrogenase, involved in the formation of lactic acid from glucose, was found to have three times the activity in the early-reproducing parasites as in the nonreproducing, ablastin-inhibited ones (12). Various data suggested that ablastin does not directly affect the enzyme level within the trypanosomes.

Changes in the physiological integrity of the cell membrane of *T. lewisi* have been reported to occur from the action of rat antiserum (16). Organisms exposed to antiserum were rapidly penetrated by tetracycline, whereas the drug was completely excluded in normal serum. In addition, leaching of material absorbing at 260 mμ from the trypanosomes was more pronounced in antiserum than in normal serum.

Warburg studies on *T. vivax* of cattle demonstrated that serum containing specific antibody inhibited the parasite's respiratory rate and that the degree of inhibition of oxygen consumption appeared to be related to the titer of antibody present (13). Although heating the serum diminished the activity of the antibody in this system, it did not destroy it (14). Fulton and Spooner (21) similarly found that human sera containing dye test antibodies reduced the respiration of *Toxoplasma gondii* as compared to normal serum.

Recently Strannegård, Lund, and Lycke (74) have reported on the effect of *Toxoplasma* antibodies and normal serum factors on the respiration of *Toxoplasma* by means of an ampulla diver technique. The respiration-inhibiting effect of antibody was found to require the presence of properdin and a high concentration of fresh human serum. The

development of morphologic alterations in the parasites was not directly correlated to inhibited respiration. Morphologic alternations and inhibition of cell penetration by *Toxoplasma* can be induced by antibody plus properdin, in the absence of demonstrable complement components (73).

The effect of dye test antibody on *T. gondii* and the mechanism of action of the dye test (54) have been the subject of considerable controversy, but it is quite apparent from electron-micrograph and cytochemical studies that morphological and structural changes are induced. Early investigations with regular and phase microscopy described swelling followed by partial lysis and cytoplasmic expulsion (35, 49). Electron micrograph studies on sectioned organisms (4) have revealed the destruction of organelles such as conoid, toxonemes, and ultimately the nucleus; these effects were interpreted as due to the penetration of antibody into the cell. On the basis of electron-microscopical (5) and cytochemical studies (32) it has been suggested that the ribonucleic acid normally present in *T. gondii* disappears or becomes undemonstrable owing to depolymerization, as a result of the action of dye test antibodies.

Toxoplasma exposed to ferritin-conjugated antibody and examined by electron microscopy were found to have a high concentration of ferritin particles on their surface (36). However, organisms contained within intact vacuoles of a host cell remained unlabeled by this procedure. Antibody was arrested at the limiting membrane of the vacuole, which accounts for the protection of *Toxoplasma* against antibody action.

In an attempt to explain stunting and inhibition of egg-laying in *N. braziliensis*, Chandler (6) proposed that the protective antibodies formed by the host acted as antienzymes against the enzymes produced by the worm for invasion and for its nutrition. Thorson (80, 83) ultimately demonstrated that secretions and excretions liberated by the worm *in vitro* were antigenic and contained lipolytic activity. Immune serum prepared against these materials almost completely inhibited the lipolytic activity of the metabolite preparations. Similarly, serum from a dog immune to *Ancylostoma caninum* inhibited the proteolytic activity present in esophageal extracts obtained from the worm (82). The extract was used to immunize puppies, and stunted worms were produced on challenge infection (83). Dusanic (17) obtained evidence to indicate that lactic dehydrogenase is secreted by the worm during infection and stimulates the production of specific antibodies in the rabbit. In this regard, it is of interest that Henion, Mansour, and Bueding (23) found the lactic dehydrogenases of *S. mansoni*, *S. japonicum*, and *S. haematobium* to be inhibited by an antiserum against the *S. mansoni* enzyme. The same immune serum had no effect on another glycolytic enzyme from *S. mansoni*, phosphohexose isomerase. In a somewhat different approach, immune serum to *N. braziliensis* was found to inhibit the oxygen consumption of infective third-stage larvae, but only slight activity was apparent against parasitic third-stage organisms, which may indicate some stage specificity (60).

Remarks

In the course of this discussion I have attempted to depict the spectrum of effects produced by immune responses on animal parasites. Of fundamental importance is recognition of the fact that in those instances in which protective immunity develops, the underlying mechanisms for the most part remain obscure. Particular *in vitro* effects elicited by a specific antigen-antibody reaction, such as formation of precipitates or immobilization, may be of temporary consequence only, causing little permanent injury to the organism. However, the potential importance of these reactions *in vivo* should not be too readily dismissed. As Jackson (27) has pointed out, "worms cannot be swept out of a closed glass vessel containing antibody as they can from the gut of an immune host."

It is obvious, however, that physiological and biochemical alterations induced by the

immune response may play a highly significant role not only in the growth and differentiation of parasites, but in their ultimate rejection by the host. In view of the very small number of studies existing in this area, the field affords many intriguing research opportunities.

Many of the studies that have been concerned with the effects of antibody action on parasites have arisen from an interest in exploiting a particular phenomenon as a diagnostic test for infection. Far fewer have been devoted to a detailed examination of the basic mechanisms of these reactions. Yet it is quite clear that such investigations are urgently needed if we are to gain a full understanding of protective immunity.

New advances in fundamental immunology, cell and cancer immunobiology, and developmental embryology obviously need application to parasitological problems. For example, the more complete understanding recently obtained of the membrane defects of red blood cells caused by complement lysis (53) may have an important bearing on the problem of lytic destruction of trypanosomes and other parasites known to be killed by such antibody action. There is also much in common between inhibition of development and stunting of helminths, and embryological developmental studies of higher organisms in which repression of organ growth may occur from the action of specific antiserum (18). Unicellular and multicellular parasites offer valuable tools with which to investigate many parameters of antibody effects on cells, and it is apparent that truly systematic exploitation of them has been relatively rare.

Moderator: May I ask Dr. Borsos to initiate the discussion.

DISCUSSION

Tibor Borsos

The study of immunity of parasites by parasitologists is a sort of three-way system. We have the parasite on the one hand, the host on the other hand, and the parasitologist on a third hand. Today we are discussing what the parasitologist should do when he is confronted with a parasite and a host in the presence of an immunologist. I hope that the somewhat pessimistic remarks made by the immunologists are not inhibitory on the parasitologists.

First I should like to discuss some of the problems of antigen separation and identification by immunological techniques.

It seems to me that the most important antigens in terms of the host's defense against the parasite or any other kind of invading organism ought to be the surface antigen of the invading organism. These are, after all, the very first antigens that come in contact with the host. Yet surface antigens are very hard to deal with, because they are difficult to extract in a form that still corresponds to the structural identity as it was in the intact surface of the parasite.

If one exposes a parasite to ethanol and all sorts of reagents, organic and inorganic, one gets something that one dries down and calls an antigen and puts into an animal to get an antibody. Can one expect that antigens treated in this way still correspond to the antigenic structure which was present in the intact host? Even though we may get a reaction between the antibody and the host in the long run, there is little chance that the integrity of the molecule as it was *in situ*—that is, in a three-dimensional geometrical area of other antigens—still exists.

I think that this point should be remembered in view of the different classes of immunoglobulins that may be generated against a given antigen. This is especially important because, as Dr. Kagan mentioned, antibodies of the different classes of immunoglobulins have different efficiencies in terms of agglutination, complement fixation, and other reactions. We have been studying the complexity of cell surface antigen-antibody interaction, using the model referred to by Dr. Taliaferro and also other models, including human red cells and their iso antibodies. We have been able to show that, in addition to immunoglobulin class, the geometry of the antigen is also important in the action of antibodies on cell surface antigen.

Let me elaborate on this point a little. IgM molecules are highly efficient both in agglutination and in complement fixation. IgM molecules have at least five reactive sites. By this fact alone, the avidity of this molecule differs from that of an IgG molecule, which has only two reactive sites. In addition, one IgM molecule has ten heavy chains, the chains that are involved in complement fixation. One IgG molecule has only two heavy chains. The IgM molecule can be looked upon as an aggregation, and I use the word very carefully here —I do not want to imply that it is aggregated IgG—whose effectiveness lies in its being "pre-aggregated." We know that one IgG molecule cannot fix complement; at least two must be present at the same antigenic site. We do not

know how close the antigen sites have to be, but certainly the two IgG molecules must land close enough to one another to form an effective unit, a unit that can fix complement. Possibly this is the unit that is also able to induce some of the other reactions indicated by Dr. Biro.

Now, if the generation of a unit of IgG—a doublet, as we call it—is a random process, the number and geometry of antigenic sites will determine whether a doublet can be formed. If there are very few antigenic sites, and they are all close together, the generation of doublets will be very efficient. If there are a very large number of antigenic sites distributed widely over the cell's surfaces, then the chance of doublet formation is virtually nil, unless thousands of molecules of the IgG molecule are supplied. In contrast, a single IgM is capable of initiating complement fixation under either of these conditions.

So depending upon how we handle these antigens, we may get shifts in the efficiencies of antibodies. If the A substance of the human red cell is studied—it is a very convenient antigen to study because a soluble form of it can be obtained and it can be had on a cell surface—the inefficiency of complement fixation at cell surfaces by the anti-A substance IgG molecule can be shown. In contrast, complement fixation by soluble A substance and anti-A-substance IgG antibody is very efficient. The difference in efficiency lies in our ability to manipulate the fluid phase concentration of both the A substance and the IgG at our convenience. Under ordinary conditions we cannot manipulate the concentration of A substance on a cell surface at our convenience; we can only manipulate the cell concentration. The very same problem faces parasitologists. They cannot manipulate antigen concentration on the surface of the parasite, but they can manipulate the concentration of the extracted antigen. Thus the extraction of antigens may alter not only their secondary or tertiary structures but also their geometric positions in space.

A further difficulty arises from the fact that to the same antigen can be produced antibodies belonging to more than one class of immunoglobulin. These immunoglobulins, as was said earlier, may manifest different activities. Depending on the ratios of the different antibodies (immunoglobulins) in a given antiserum, one antibody may interfere with the action of another; this interference may be the result of steric hindrance of neighboring sites, competition for the same site, differences in aridity, and so on; thus, different results may be obtained, depending on experimental conditions. These conditions include, among others, the dilution of antiserum, the source of antiserum, and the time of removal of antiserum after antigenic stimulation of an animal. All these factors may contribute to a great deal of difficulty in interpreting results.

I want to direct my final remarks to what Dr. Dubos was saying. I think that Dr. Biro's distinction is well made.

I do not want to resurrect Metchnikoff, Ehrlich, and the theories of humoral versus cellular defense mechanisms, but there are extracellular substances such as antibody and complement that by themselves are capable of killing certain invading organisms; the role of the macrophage in this instance seems to be the elimination of debris left behind. The first demonstration of the effectiveness of humoral factors in killing bacteria *in vivo* was by Pfeiffer in 1894.

I also want to point out that often the last refuge of a parasite is the inside of a cell. A number of viruses and other parasites survive for many years inside cells in the presence of antibodies. Some of these parasites, indeed, survive inside cells where there are lysosomes. I don't want to say that lysosomes and macrophages are not important—that is not my point. The point I am raising is that we have more than one kind of defense mechanism to worry about and that one should not be ignored in favor of the other.

Moderator: I believe Dr. von Lichtenberg has a comment to make.

Lichtenberg: With your permission, I should like to show some slides that have a bearing on the remarks of Dr. Smithers and Dr. Weinstein

on the surface characteristics of the different life phases of *Schistosoma mansoni.* These are important, I thought, because they illustrate some of the antigens that the host antibodies—about which there has been so much discussion here—are reacting to.

My first figure shows an electron micrograph of a cercaria of *Schistosoma mansoni* at the point of attachment of the body to the tail, represented by the gray zone in the middle. As you see, the outer surface of the cercaria is covered by finely fibrillary material that is noncytoplasmic and constitutes a glycoprotein that has been called the "cercarial envelope." This has been observed by Dr. Stirewalt and by others. In addition, Dr. Stirewalt showed that the reaction with host antibody took place with just this material, and you can visualize here how such a reaction would appear in the low-power micrograph of the cercarienhüllen reaction that was shown this morning by Dr. Smithers.

The integument itself—the living cytoplasm of the cercaria—is organized very much like that of the succeeding life form, which will be shown in the second figure. This represents the schistosomulum at its lung stage. This stage is seen to lack a mucopolysaccharidal envelope—which, by the way, is not a product of the parasite integument but comes from an unknown source in the cercariae. Here in the schistosomulum we can see a very active cytoplasmic symplasium with a unit membrane on its outside that—at least in this static picture—appears to be engaging in a lot of movement, invagination, and resicle formation similar to pirocytosis. The third figure is an electron micrograph of an adult organism seen by "thick section," showing details of the spines and the dorsal integumental surface. We have incubated this worm in a medium containing horseradish peroxidase, a protein macromolecule that was used by Karnovski and others to study the transport of macromolecules across endothelial surfaces. Here it can be seen that the integument of the schistosome is in fact taking up the macromolecular material labeled by the benzidine stain, and that this material is also appearing in the somatic cells of the organism.

Therefore, it would seem that an antibody directed against the surface material of the cercaria would not be effective against schistosomula or adult worms. Further, one would think, as Dr. Smithers has said, that perhaps the living surface of the adult schistosome would be able to treat host proteins, including any antigen-antibody complexes present, as food, and ingest and metabolize them.

Remington: Dr. Weinstein mentioned that many of the studies on delayed hypersensitivity in nematode infections reported in the past few years leave much to be desired. It appears that these workers have equated cellular resistance or cellular immunity to delayed hypersensitivity and the role of the lymphocyte or immunocompetent cell. In some experimental models, "cellular resistance" develops simultaneously with the appearance of delayed hypersensitivity to certain antigens of the organism. *In vitro* studies such as those reported by John David and his colleagues have demonstrated that the migration of macrophages is inhibted in the presence of the antigens to which the host is hypersensitive. This inhibition of macrophage migration appears to be dictated or mediated by lymphocytes of the immunized animal. But the relationship of these findings to actual immunity is unclear. Mackaness has demonstrated "immunity" of peritoneal macrophages *in vitro* and *in vivo* in animals immunized with live listeria or salmonella. In such animals there is no good correlation of circulating antibody levels with immunity, and immunization with dead bacteria does not confer immunity. Most important is the fact that animals immune to listeria are also immune to challenge with virulent strains of salmonella. As Dr. Dubos has said, nonspecific stimulation of the production of lysosomal particles within macrophages can be produced even with glycogen. By increasing the numbers of lysosomal particles in these cells, the macrophages appear to have an enhanced "nonspecific" resistance to bacterial invasion.

There is one other point I should like to mention in regard to the effect of the immune re-

FIGURE 1. Cercaria of S. *mansoni* at point of attachment of body to tail (gray zone in middle).

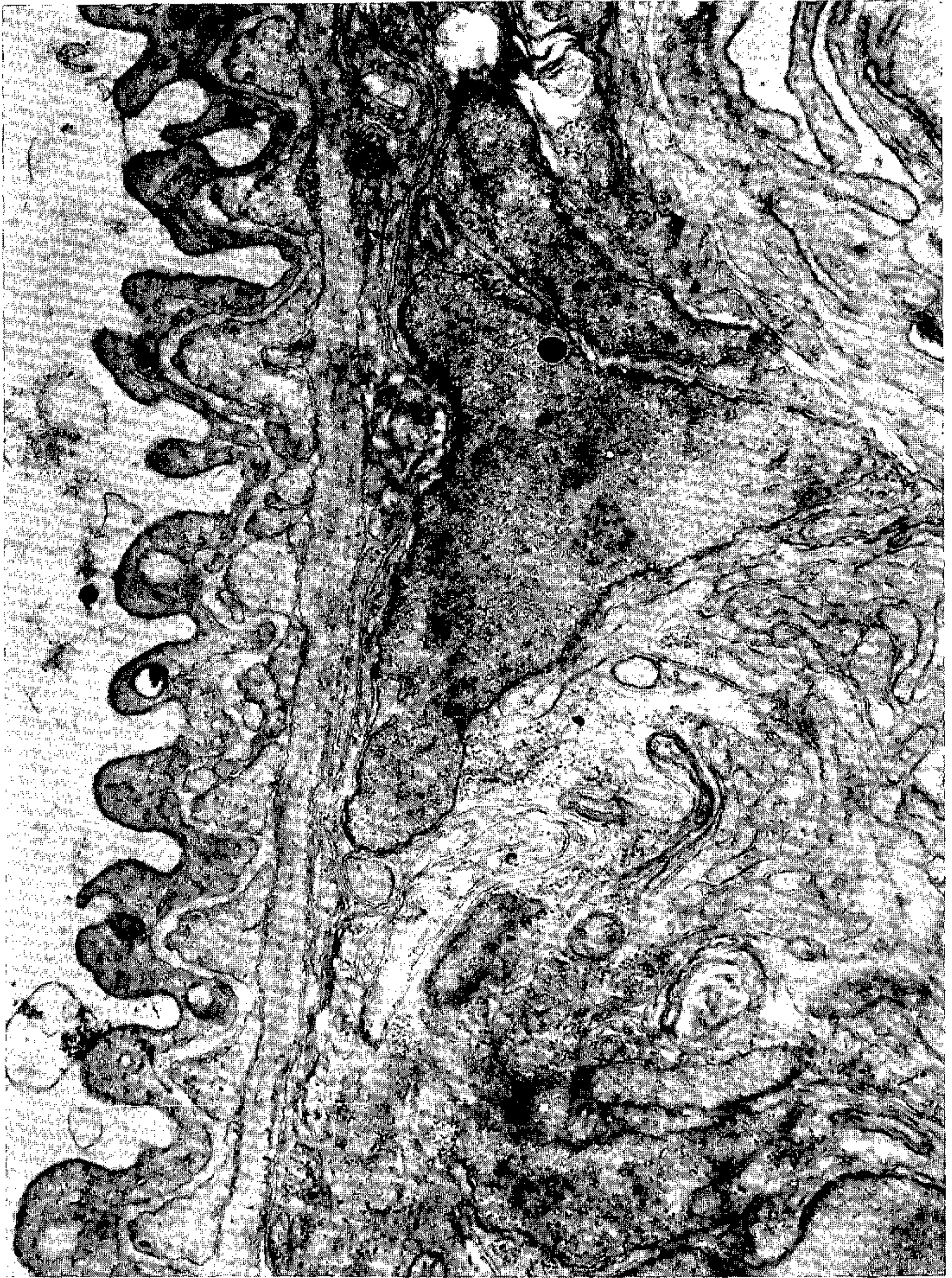

FIGURE 2. Schistosomulum at its lung stage.

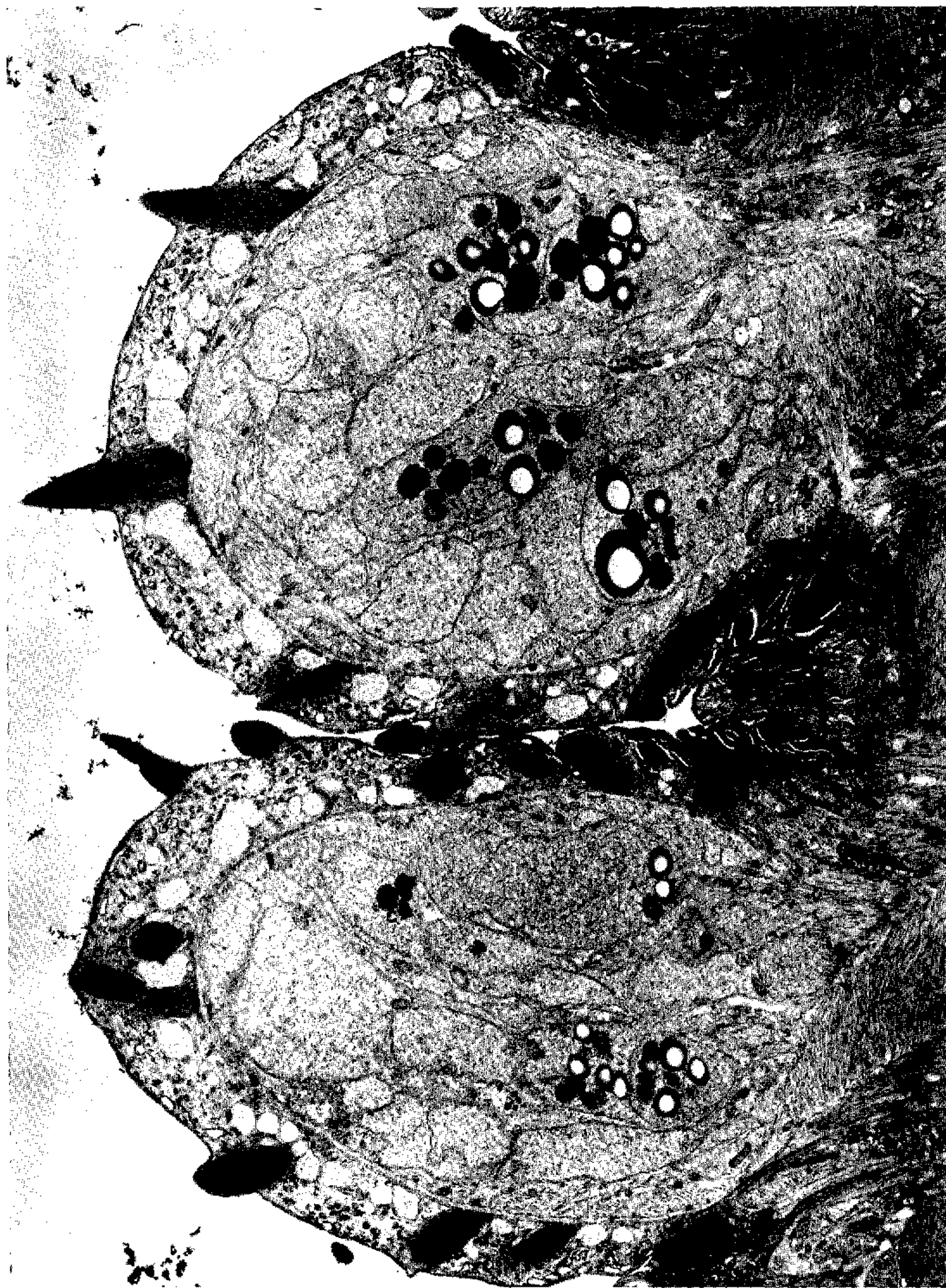

FIGURE 3. Adult organism of S. *mansoni* seen by "thick section."

sponse on parasites. Dr. Biro mentioned in a roundabout way the difficulties encountered in cellular and humoral immunity to virus infections. I suspect that he was indirectly referring to agents such as interferon. We have recently demonstrated the stimulation of interferon production by toxoplasma. This discovery raises the question of the role of interferon in infection with other intracellular parasites. As yet, we have been unable to demonstrate that the interferon resulting from infection with toxoplasma or that produced by viral infection can prevent or even suppress toxoplasma infection. However, the interferon resulting from infection with toxoplasma can definitely inhibit viral infection, as has been demonstrated in our experiments with mengo virus in mice.

Moderator: We come now to the last topic on our program, presented by Dr. von Lichtenberg.

THE BILHARZIAL PSEUDOTUBERCLE: A MODEL OF THE IMMUNOPATHOLOGY OF GRANULOMA FORMATION*

Franz C. von Lichtenberg

Introduction

Host reactions in parasitic infections are ruled by the same immunological principles that regulate all other infectious diseases (6). Therefore, if any of their features appear unique, they must be derived from the specific biological properties of parasites and their products. By the same token, analysis of these variegated responses, so different from classical models, will help to enlarge our perspective of the total range of host defense mechanisms and their interactions in disease.

Schistosome flukes are among the largest tissue-dwelling agents and secrete or excrete a variety of enzyme-containing and antigenic products (4, 5, 17, 24, 51, 55, 59). Their mammalian phase culminates with intense and sustained reproductive activity inside host veins. Yet in their natural hosts they rarely produce critical illness and characteristically achieve a stable and long-lasting host-parasite balance that may remain entirely subclinical or shift toward eventual host disability or death. In *Schistosoma mansoni* infection, the following key events have been identified through experimental study:

About 28 days after primary exposure the flukes that have survived penetration and migration begin reaching sexual maturity; oviposition starts and through about the tenth week of infection the number of eggs deposited in host tissue rapidly increases. Egg destruction, a slower process, lags behind at first, but sometime around the fourth month of infection it attains a rate equivalent to deposition, so that egg turnover in host tissue stabilizes (7). Even before oviposition, antigen is released by the maturing worms; thus immunofluorescent antibody can be detected by the third week (21) and, in massive murine infection, circulating antigen is discovered by immunoelectrophoresis on the 26th day (4). Likewise, immediately after the deposition of each new schistosome egg, immunofluorescent stainable antigen is diffused for a limited period of time, probably aggregating a substantial antigen influx in early infection (27). However, during this acute phase, which may be marked by disseminated lymphoreticular activation (21, 37) and by systemic illness (13), multiple antibodies are formed (25, 51) and their titer ascends steeply, as does the degree of cellular sensitization (21). As a result, the disposal of schistosome antigen becomes accelerated and increasingly efficient, as will be shown in greater detail below. By the time egg turnover becomes constant, symptomatic remission has ushered in the chronic phase of the infection (13). By this time, too, flukes residing in the mesenteric radicles have completed their differentiation and acquired the capacity to assimilate and transport various metabolites and macromolecules across their integument (54). Whether for this or for other reasons, their living surface becomes inured to surrounding host cells and

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antibodies (which readily attack any damaged worms). Similarly, miracidia protected by intact egg shells and by the release of their secretion maintain an unimpaired 21- to 32-day viability period (38, 60) even while surrounded by host cell granulomas. Finally the host becomes resistant to reinfection, although this varies in degree by species and experiment design (48). In the natural hosts of schistosomes, immunity is feeble compared to that of species capable of self-cure (57), but it is likely that, even in man, the worms do not go on increasing indefinitely and a relatively stable ceiling or—under favorable conditions—a decline of the infection is eventually reached (41).

Thus, in schistosomiasis, host-parasite balance is the result of mutually counteravailing defense mechanisms of host and parasite, rather than of low parasite virulence or suppressed host reactivity. During the chronic phase of the infection, the balance is rarely disturbed except by such events as massive "toxic" superinfection, ectopic lesions in vital areas, or intercurrent pathology. In the long run, gradual and repetitive formation and resolution of pseudotubercles leads to structural distortion of organs and to impaired flow in sensitive vascular territories. This late and sometimes life-threatening pathology is poorly understood, but there is evidence that it is related to high egg burdens over long periods of time (9, 50).

The immunopathology of schistosomiasis presents two major problems: the factors that determine acquired resistance and those that play a role in defense against established infection. While the former have been studied in more detail (28), the latter are of at least equal significance. Part of our research has therefore dealt with the disposal of schistosome egg antigen by the mammalian host. These studies and the pertinent hypotheses will be summarized below.

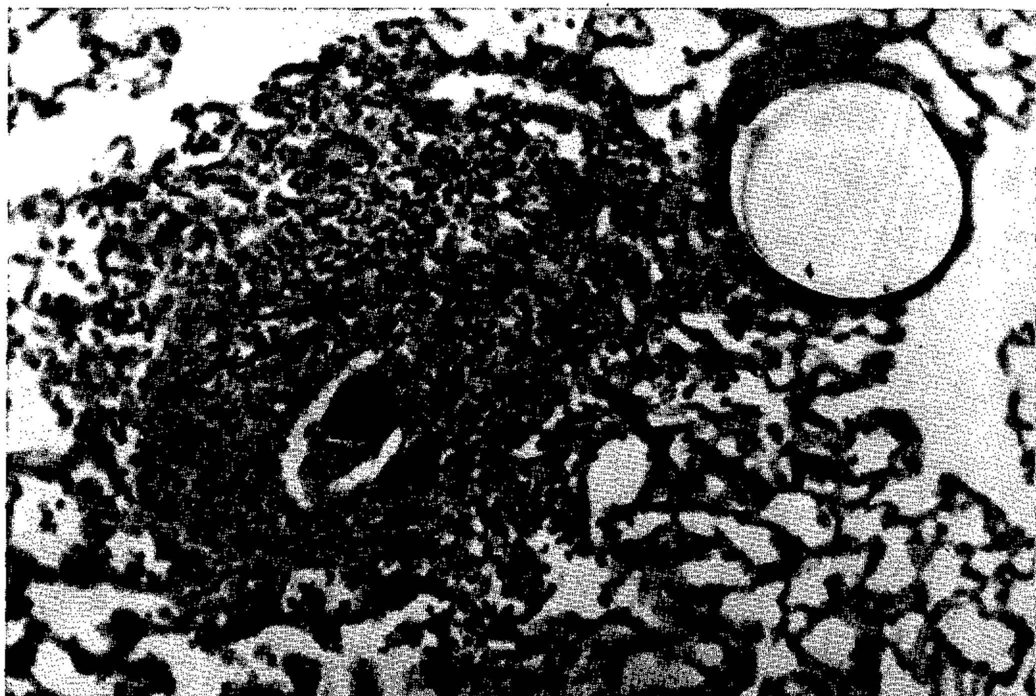

FIGURE 1. Mouse lung, 32 days after intravenous injection of *Schistosoma mansoni* eggs and of di-vinyl-benzene-copolymer beads (Hematoxylin-Eosin, x 240). The schistosome pseudotubercle is at its peak development, whereas the reaction to the plastic bead is reduced to a thin fibrous sheath.

Studies on the schistosome pseudotubercle

Production of experimental pseudotubercles

When viable *S. mansoni* eggs obtained from mouse livers (11) are injected intravenously into unsensitized mice, they disperse in the lung, forming discrete pseudotubercles that can be sequentially measured and compared with reactions to control particles. Purified egg suspensions are heterogeneous as to age and preservation of individual ova and have lost some antigenicity, but experimental granulomas, while averaging about 150 μ less in mean diameter, are otherwise similar to their natural counterparts in location and cell composition. After an initial lag of all response, primary experimental pseudotubercles increase to their peak size within 16 to 32 days, then slowly involute and heal, probably earlier than the sixth month after onset (26). *Ascaris suis* granulomas are similar in course, but somewhat faster in onset and healing than schistosome granulomas, and both differ markedly from reactions to insoluble polyvinyl spheres, which tend to terminate early by the formation of a thin, fibrous sheath around each plastic bead (26), as may be seen in Figure 1.

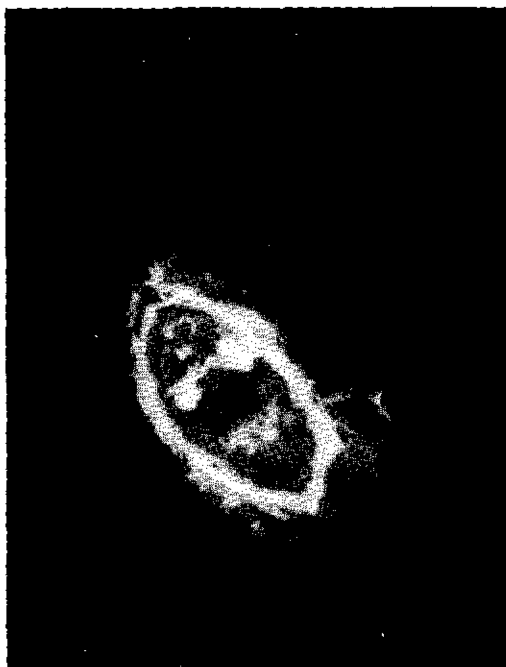

FIGURE 2. Mouse liver, 8 weeks after cercarial infection with *S. mansoni;* cryostat section stained with fluorescein-conjugated immune *Mastomys* globulin by the direct Coons technique (x 370). The paired miracidial cephalic glands and the antigen deposits on the inner and outer eggshell surface are intensely positive. Antigen is seen in adjacent granuloma cells, fading toward the periphery. Some granulocytes show nonspecific fluorescence.

Stainable schistosome egg antigen in pseudotubercles

By means of the Coons immunofluorescent technique with its proper controls (27), it can be shown that in the unsensitized host an amorphous, specifically stainable product is diffused from eggs for at least 24 hours following injection, after which the material is taken up by phagocytes congregated around the egg. By the fourth day stainable antigen, contrasting vividly with the orange-yellow autofluorescence of the egg shell, is deposited on both its inner and its outer surfaces and in cytoplasmic particles within granuloma cells. Thus evidence of antigen diffusion is now replaced by a visual image of "antigen sequestration" (Figure 2). From the fourth through the eighth day, stainable antigen is rapidly depleted, but fine, powdery particles in the miracidia and host cells remain demonstrable for 60 to 70 days—past the onset of involution of the pseudotubercle. This sequence, originally referred to as "rapid" and "slow" antigen disappearance, is probably the composite result of ending antigen generation in the presence of continuing catabolism (27). The homologous, immunofluorescent circulating antibody does not become detectable until two weeks after egg injection (21); thus, as in the case of soluble protein antigen (58), catabolism precedes detectable antibody formation.

When naturally infected mouse-liver tissue is stained with the immunofluorescent technique, most granuloma centers fluoresce selectively as if lit by a magic lantern, and the various sequential phases seen after egg injection all appear concurrently. In the best preparations, the miracidial cephalic glands and cortex are stained intensely, together with the glassy antigen deposits along the egg shells (Figure

2). When specimens are demounted, washed, and restained, all these deposits are positive with the periodic acid-Schiff stain (27).

Reactions to separate egg components

Pure miracidia (31) when injected intravenously, cause a mild leukotactic response (Figure 3) and disappear without trace within 48 hours. Diffusion of stainable antigen occurs during the first hours after injection, as with whole eggs, but antigen sequestration and granuloma formation do not follow. Pure egg shells, whether obtained by maceration or by sonication-centrifugation (31), cause an inflammatory cell reaction lasting somewhat over two weeks. Some of these egg shells retain traces of Coons-stainable material, and a few eosinophils and epithelioid cells participate in the early cell response; later, as in the case of the plastic spherules, the shells remain ensheathed by a few stereotactic giant cells or histiocytes (Figure 4). The reactions to miracidia or egg shells do not qualify as true pseudotubercles, and even their aggregate size and duration are less than the corresponding reaction to intact eggs, whether viable or heat-killed (Figure 5). Live and dead intact ova cause the formation of pseudotubercles similar in cell composition and reaction profile, but the reaction to autoclaved whole eggs is less in size and duration than that to viable eggs (Figure 5) (31). When compared by the immunofluorescent technique, viable eggs are found to generate amorphous stainable antigen during at least the first four days of their residence in host tissue, while heat-killed eggs show a gradual depletion of this material, together with a bluish-white autofluorescence suggestive of protein denaturation. Nevertheless, antigen diffusion and its uptake by host cells can be observed in both (27).

Hypothesis of antigen sequestration

According to this evidence, both miracidial antigen and relatively inert shell material (29)

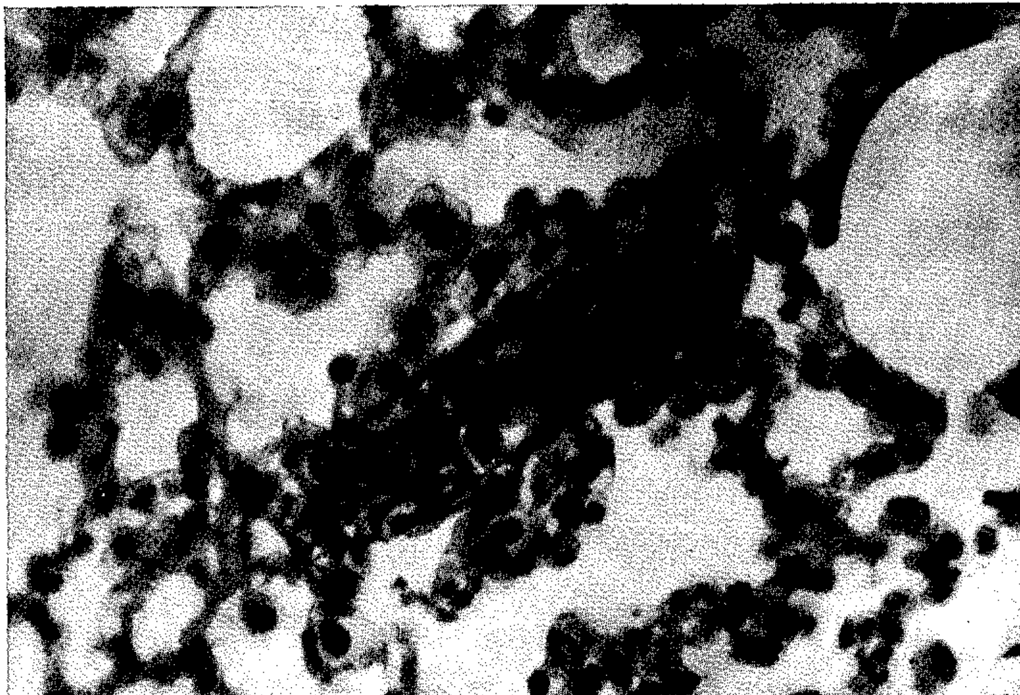

FIGURE 3. Mouse lung, 24 hours after injection of pure miracidia; periodic acid-Schiff stain (x 420). The strongly positive miracidium, impacted in a capillary, is disintegrating and has attracted numerous leukocytes to its vicinity. There is no granuloma formation.

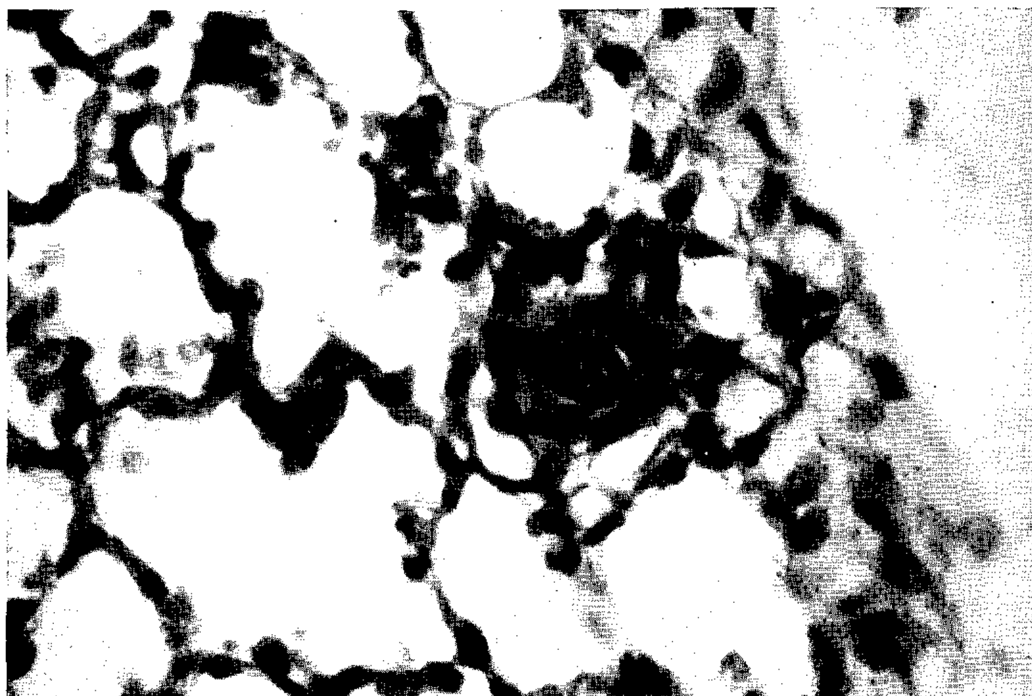

FIGURE 4. Mouse lung, 64 days after injection of purified egg shells obtained by sonication-centrifugation (Hematoxylin-Eosin, x 420). The egg appears as a basophilic spiral surrounded by a few histiocytes. This represents the residual stage of the reaction.

are required for granuloma formation, but the miracidium must be contained in the intact egg shell so that antigen is gradually and continuously released rather than quickly dissipated. The larger size of pseudotubercles caused by viable as against heat-killed eggs is explained by their more generous endowment with immunofluorescent diffusible antigen and suggests that this material is a miracidial secretion product. Direct evidence of miracidial secretion and of submicroscopic pores in schistosome egg shells will be supplied below to show that diffusible antigen can indeed be gradually released by schistosome eggs much as drugs are released from so-called "spansules."

A close analogy is evident when the pseudotubercle is compared with the "adjuvant effect" —the enhancement of local cell reaction and antibody formation resulting when diffusible antigen is injected in the form of oil- or wax-coated particles. In both cases, soluble antigen at first diffuses freely, but as soon as host phagocytes become nonspecifically attracted to the particles these cells take up newly emerging antigen on contact provided it is macromolecular or attached to a phagocytable carrier. Continued antigen release then results in antigen sequestration and in primary granuloma formation. This sequence can be triggered in the absence of host sensitization, depending only on the manner in which diffusible antigen becomes available to host cells *in situ.* Eventually a gradient of antigen concentration develops from the center to the periphery of the primary granuloma, systemic antigen diffusion is reduced, and, as circulating antibody makes its appearance, the host becomes immunologically responsive.

Effect of sensitization on granulogenesis

When mice are sensitized intraperitoneally with S. *mansoni* eggs and are then challenged intravenously after suitable intervals, a modified, secondary granulomatous response occurs,

which has been analyzed in some detail by our group, including the tragically deceased Dr. Ramón Gómez Mazzei (18) of Asunción, Paraguay, and by K. S. Warren and collaborators (63). Some of these studies are still in progress at this writing.

Accelerated and enhanced granuloma formation. This effect was first reported as a twofold or greater enhancement of granuloma size on the fourth day after challenge in 24-hour- and 2-week-old mice sensitized and challenged with *Ascaris suis* eggs (39). Later, Gómez Mazzei showed in experimental S. *mansoni* pseudotubercles that both the total cell and the eosinophil response were markedly enhanced by the second day, and he was able to demonstrate the specificity of this effect (18). Warren confirmed this further (63) and has shown that sensitization is detectable after 24 hours by quantitating the proportion of eggs showing any cell response whatsoever (personal communication). With this method, he and his group are currently exploring the factors in the induction, suppression, and passive transmission of the secondary granulomatous response that will be discussed below.

Accelerated antigen disappearance. Whether accelerated secondary cell response results in earlier antigen sequestration has not yet been explored, but accelerated antigen destruction is well documented. In sensitized hosts, Coons-stainable antigen is found to be virtually extinguished by the 32nd day after challenge, as against the 70th day in unsensitized subjects (27); in fact, the proportion of granulomas containing stainable antigen is already signifi-

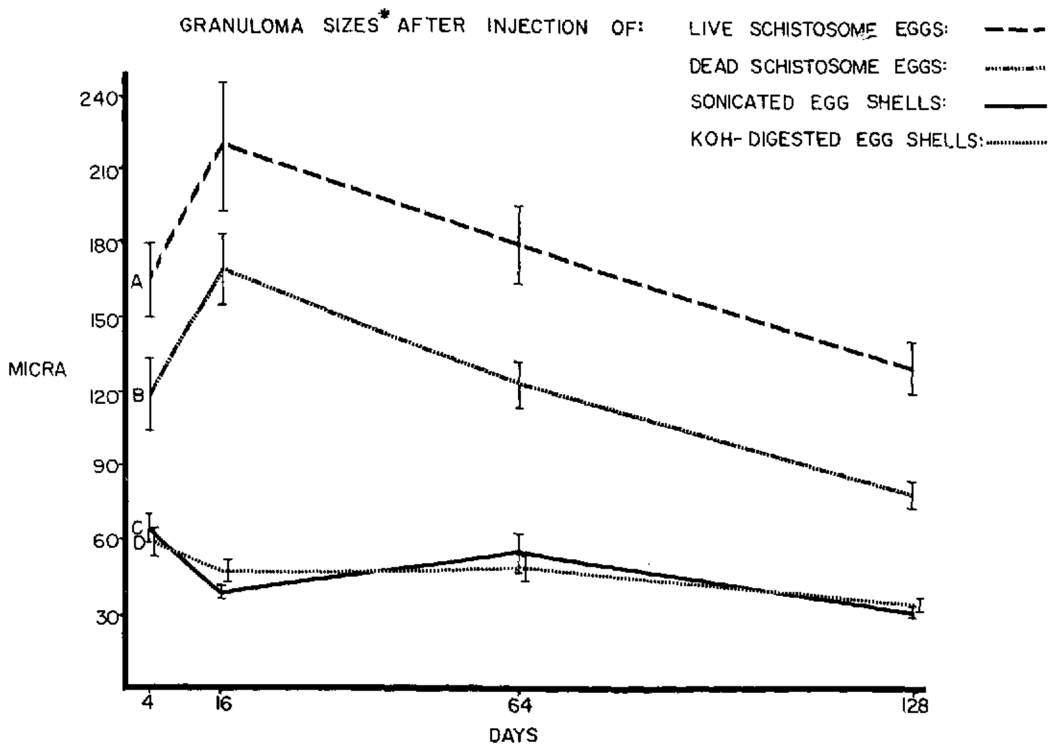

FIGURE 5. Graphic representation of cellular reaction diameters around viable and heat-killed S. *mansoni* eggs and around two types of purified egg-shell preparations (see text), at successive intervals after intravenous injection into unsensitized mice.

cantly reduced by the eighth day after challenge (44). Using this experimental endpoint, Peterson showed that the antigen in pseudotubercles is sensitizing *in vivo* as long as it can be visualized there by immunofluorescence and irrespective of its coexistence with circulating antibody in the same host (44). While an anamnestic antibody response to repeated egg challenge has not yet been studied, it has been demonstrated in analogous experimental situations (21).

Reduced total duration of pseudotubercles. Gómez Mazzei noted that, although initially larger, secondary granulomas actually became smaller than those of unsensitized controls by 70 days after challenge, and that fewer egg shells were detectable at that time. Since this experiment had not been repeated, he mentioned it without descriptive detail (18). Warren's results also showed a steeper fall in the size of secondary granulomas between the 16th and 32nd days after challenge than occurs in primary granulomas (63).

Increase of concomitant alterations. In all these experiments, including the very earliest, systemic alterations were found to accompany granuloma formation. In secondary response, pulmonary alveolitis (26), lymphoid cell mantling of blood vessels (39), and intimal proliferation in pulmonary arterioles were found to be increased (26, 39). When schistosome eggs were injected prior to cercarial infection, splenomegaly was increased over that found in the controls (33). When viable schistosome eggs were given by repeated intravenous injection up to 14 times, a pulmonary arteritis was produced in mice that resembled human bilharzial pulmonary arteritis in that hiatuses of the elastica layer and multiple channel formation in arterial lumina (Figure 6) were present (2);

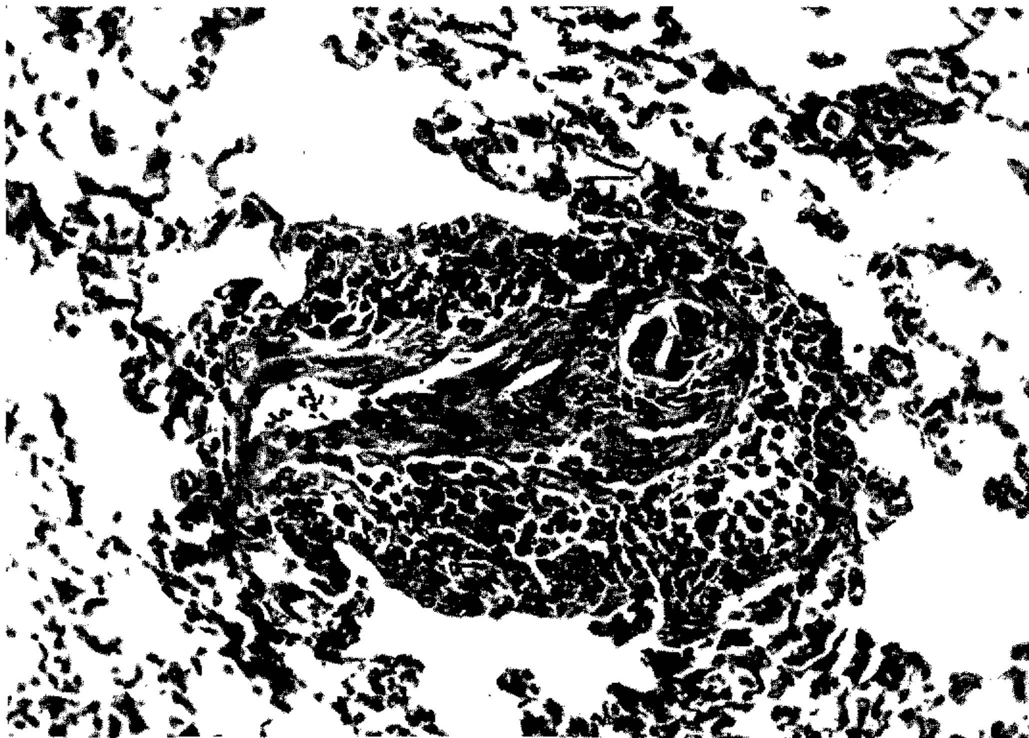

FIGURE 6. Mouse lung after 4 successive monthly injections of viable *S. mansoni* eggs (Hematoxylin-Eosin, x 220). The arteriole shows marked intimal proliferation with multiple cleft-like lumina, appears thickened and surrounded by a dense lymphoid cell infiltrate. An involuting pseudotubercle containing an egg shell is seen in the vessel wall, right of center.

however, typical angiomatoids and right ventricular hypertrophy did not appear. The design of these unpublished experiments precluded statistical analysis, but the granuloma size did not seem to increase beyond the time of the third or fourth successive challenge—that is, the second to fourth month of observation.

Pseudotubercles in natural infection

Compared to any of the preceding models, the acute phase of cercaria-induced schistosome infection is a condition of maximal host reactivity: the granuloma reaches its largest attainable size during the second to third month of infection (7), often giving rise to presinusoidal portal hypertension and to the early murine hepatosplenic syndrome first described by Warren and deWitt (64). Stainable antigen is calculated to persist for less than 34 days (27), close to the calculated maximal life span of miracidia; in view of the better preservation of eggs left *in situ* compared to those in purified suspensions, this may be at least as effective a host performance as is found in the artificially sensitized model. Circulating antibody experiences a steep, anamnestic type of rise with a fivefold or greater increment over the titers attained by a single purified-egg injection (21). Concomitant alterations, including scattered lymphoid cell infiltrates, are likewise maximal at this stage; "lymphoreticular activation" (21, 37, 45) results in hepatosplenomegaly with splenic follicular enlargement and, frequently, hyperglobulinemia (37, 45). As has been shown by Raslavicius, these manifestations are transmitted to the uninfected parabionts of schistosome-infected mice in the absence of cross-passage of schistosome eggs (45). If the portal vein is ligated to induce collateral formation and egg passage into the mouse lung, a florid pulmonary arteritis replaces the sporadic lesions usually found; this arteritis is more intense than its experimental analogue induced by repeated intravenous egg challenge (62), but neither of these models fully reproduces human bilharzial cor pulmonale.

Two additional features appear in natural schistosome infection that have not yet been observed in any other experimental model mentioned so far—central necrosis of granulomas and *in vivo* circumoval eosinophilic precipitate, also called the Hoeppli phenomenon.

Central necrosis of pseudotubercles. This lesion is most frequent in acute sublethal or lethal infection of mice and in other heavily exposed small laboratory mammals and primates (8, 32, 49). Characteristically, the necrosis is circumoval, well limited, and not as extensive as in mycobacterial infection. The necrotic zone may be eosinophilic or may contain basophilic nuclear fragments or dust. The florid type of pseudotubercle, with numerous centrally aggregated neutrophils, is probably a variant or precursor of this lesion. As Cheever has shown, maximal granuloma size in the liver decreases after the transition from the acute to the chronic stage of infection, although mean granuloma size does not clearly diminish (7), but with the waning of large, florid pseudotubercles, central necrosis also becomes rare in chronic infection.

The Hoeppli phenomenon. Stellate eosinophilic precipitates similar to those of other parasitic and fungal infections (Figure 7) have been described in schistosomiasis since before 1932 (20), although they have not always been recognized as a lesion distinct from central necrosis. Since 1954 (42), *in vitro* circumoval precipitate has been identified as an antigen-antibody complex, reactive with heterologous antiglobulin (23, 42, 47). Unlike the *in vitro* complex, the Hoeppli phenomenon requires an especially intense degree of infection and is correlated with large or rapidly accumulating egg loads in host tissues. It appears during acute schistosomiasis, most frequently between the 9th and 15th week of infection and usually in organs heavily infested with ova. No more than 10 per cent of all granulomas are affected, and none containing eggs with immature miracidia show precipitate (34). Although the immunological setting of the Hoeppli phenomenon resembles that of central necrosis, these two features do not often coincide within the same single granuloma. Detailed immunofluorescent studies of S. *mansoni*-infected livers of *Mastomys coucha*

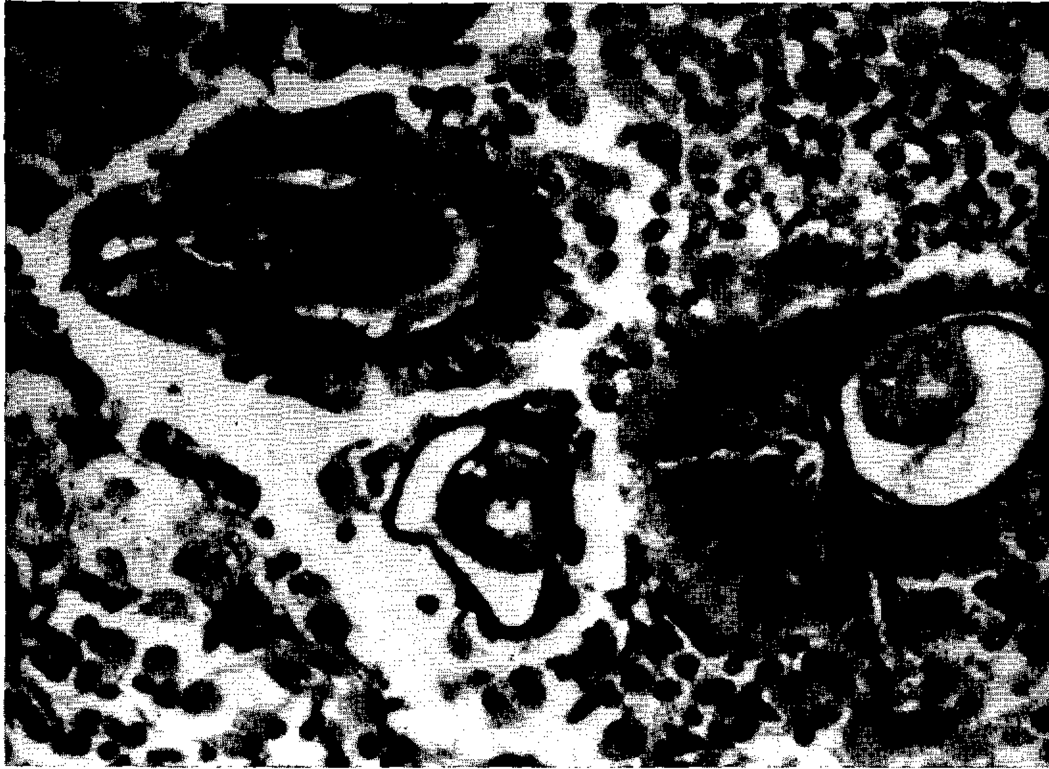

FIGURE 7. Colonic submucosa of baboon, 7 months after exposure to 1000 cercariae (Hematoxylin-Eosin, x 400). Cluster of Hoeppli phenomena in a composite granuloma with central necrosis (an unusual occurrence). Egg on left shows a mature miracidium and fully developed, spectacular Hoeppli corona. Egg on right has a degenerate miracidium and blotchy, aging Hoeppli phenomenon.

were undertaken in adjacent serial sections, to show that both antigen and fixed host globulin are present in the Hoeppli precipitate; antigen predominates in the center and the presumed antibody globulin in the peripheral zone of the complex (Figure 8). Both the Hoeppli phenomenon and the *in vitro* circumoval precipitate are formed in the outer surface of the egg shell and do not appear to affect the vitality of miracidia. Once formed, the Hoeppli precipitate matures and undergoes degradation parallel to the involution of the entire pseudotubercle (34).

Pathogenesis of the secondary granulomatous response

We have shown that in the sensitized host the events that take place in primary granuloma formation are accelerated and their total duration is reduced, which results in more efficient and quicker disposal of schistosome egg antigen; this gain is achieved at the expense of enhanced cellular response both in the pseudotubercles and systemically, and is accompanied by antibody formation. While "infectious allergy" is usually defined by the conversion of a previously negative skin test, the changes described above are probably more significant.

Just as intact eggs are required in primary pseudotubercle formation (31), the secondary response also requires sensitization with whole schistosome eggs, either viable or subjected to freezing-thawing; sonicated or mechanically disrupted eggs were found to be ineffective by Warren *et al.* (personal communication). On the other hand, homogenates of primary pseudotubercles remain sensitizing for several weeks after their onset, as has been shown by Peterson

(44). This evidence strongly supports the hypothesis of antigen sequestration and the proposed analogies between schistosome eggs and antigen-adjuvant mixtures that have been discussed earlier.

Procedures that suppress the homograft response also inhibit primary pseudotubercle formation but have little effect on secondary response. Thus primary *A. suis* granuloma formation is delayed during the first eight days post partum in mice (30), but sensitization is not abolished despite their apparent immunological immaturity (39). Warren *et al.* have successfully inhibited primary S. *mansoni* pseudotubercle formation by neonatal thymectomy (15), antilymphocytic serum (63), and a variety of immunosuppressive drugs (14).

How sensitization is mediated remains unsolved. Warren *et al.* were able to transmit the secondary granulomatous response passively by means of sensitized spleen cells, but not by immune serum (63). This experiment clearly proves the systemic nature of sensitization and suggests that it is mediated by delayed hypersensitivity, as is indicated also by the relatively slow onset of granuloma formation and the frequency of concomitant perivenular lymphoid cell infiltrates (61). On the other hand, even a single injection of schistosome eggs induces circulating antibody (21), and the early eosinophilotaxis observed in secondary granuloma formation suggests the formation of antigen-antibody complexes (18, 35). In acutely infected and highly sensitized hosts, clearcut antigen-antibody precipitates occur both *in vitro* and *in vivo* (34). I would therefore propose that both delayed hypersensitivity and circulating antibody have closely interrelated roles in mediating secondary pseudotubercle formation, and that antibody formation increases in importance proportionally to the degree of host sensitization. Until methods are found to decode this interplay, cellular and humoral factors in granulogenesis are perhaps best regarded as inseparable. This situation can be allegorically represented by the celebrated Koan riddle attributed to the Zen master Mokurai in a challenge to his pupil Toyo. "Show me the sound of two hands clapping," demands the Master, and Toyo claps his hands. "Good, now show me the sound of one hand clapping."

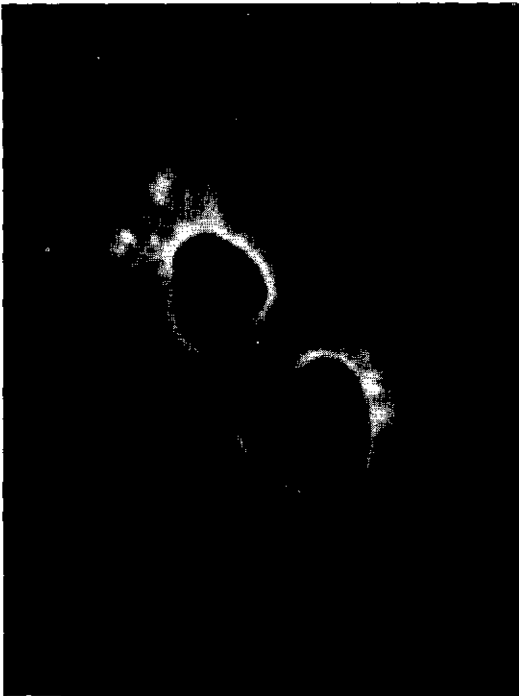

FIGURE 8. *Mastomys* liver infected for 11 weeks with *S. mansoni*; serial cryostat sections stained with rabbit-antimastomys globulin conjugate only (x 370). An apple-green halo surrounds the autofluorescent spikes of the Hoeppli phenomenon and blurs their outline. This demonstrates the predominant peripheral location of fixed host globulin in the precipitates. Adjacent sections, stained for antigen, showed the central distribution of the latter.

Perhaps a more tractable enigma is the relationship of *in vivo* precipitation and central necrosis in pseudotubercles, both of which tend to occur in highly sensitized hosts. Assuming that circulating antibody reactive with schistosome egg antigen is ordinarily not sufficient to overcome antigen excess in the granuloma center, this relationship might express itself in the customary form of phagocytic antigen sequestration. Should antibody titer rise to a level sufficient to create a zone of antigen-antibody equivalence adjacent to the mature ovum, *in vivo* precipitation would occur. In this context, precipitation can be considered a form of highly effective antigen sequestration, a concept applicable to other infections produced by

bulky organisms or colonies of organisms and accompanied by marked host sensitization (19, 36). In the light of this concept, central necrosis might have a critical humoral component, perhaps the formation of soluble antigen-antibody complexes similar to those responsible for other types of immunological cell damage such as arteritis (10, 65), but undoubtedly cellular sensitivity also plays a role (46). While in many infections central necrosis persists during the entire course of their activity, in schistosomiasis it is largely confined to the acute phase that precedes the stabilization of egg turnover in host tissues. These clues, and others mentioned earlier, deserve to be followed up by further experimental studies.

The familiar language of immunology has served us well in this discussion, by identifying useful precedents and analogies for most of the phenomena studied. However, if the pseudotubercle is to be fully understood, it must be analyzed on the biochemical and enzymatic level as well.

Antigen sources in schistosome eggs

Although the basic structure of miracidia has long been known (16), their physiology and ultrastructure are still poorly understood (22). Likewise, the chemistry and ultrastructure of schistosome egg shell is just beginning to come under scrutiny. Recently Smith has shown that egg shells of S. *mansoni,* as seen in purified suspensions, consist of an inner electron-dense layer, a wide middle zone containing submicroscopic pores, and a thin outer layer covered by "microspikes" (Figure 9). Each of these spikes shows a dense core, a light middle layer, and an outer lining formed by an array of globular subunits (unpublished) (Figure 10). Similar structures were seen by Seiti (52) and by Stenger *et al.* (56) in eggs surrounded by granulomas.

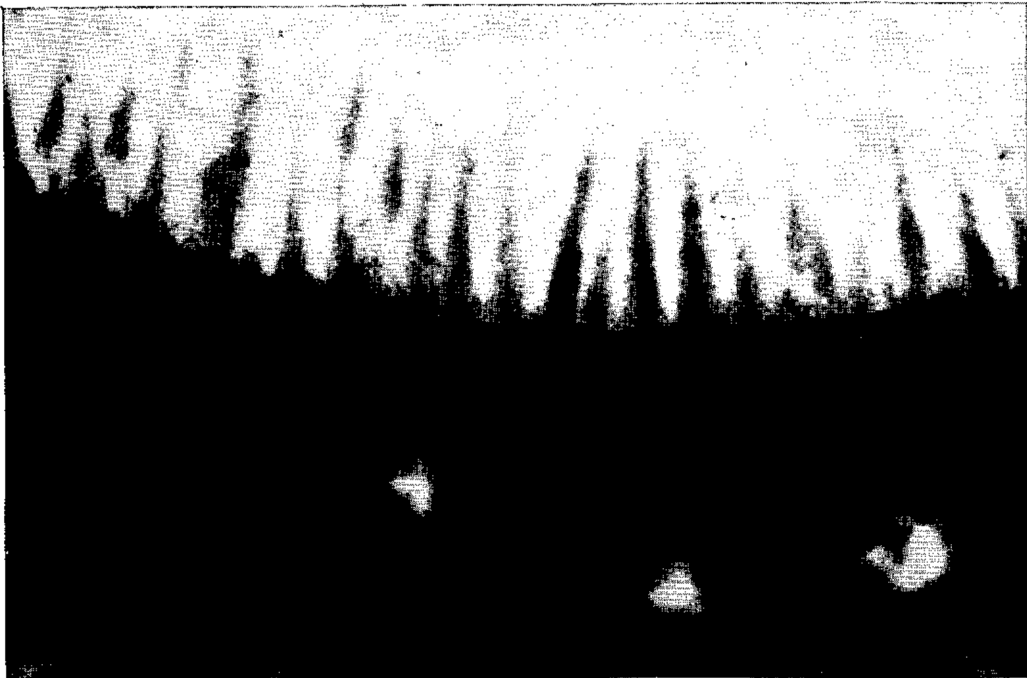

FIGURE 9. Electron micrograph of schistosome egg shell obtained from a purified suspension; glutaraldehyde-osmium fixed (x about 43,000). Shows the trilaminate egg shell structure described in text, with prominent pores in middle layer and with closely spaced superficial microspikes. The fine structure of the latter is better seen at higher magnifications.

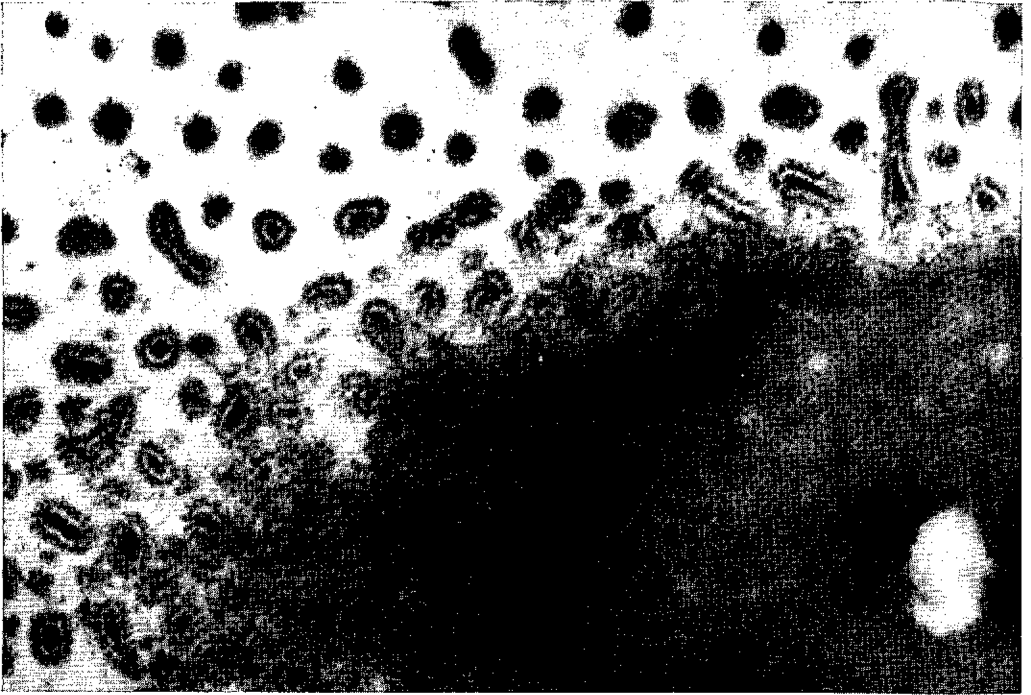

FIGURE 10. As in Figure 9, tangential section (x 80,000). Shows the dense cores and the globular arrays of the microspike membrane. A shell pore is seen in the right part of the field.

Histochemically, the miracidial cephalic glands contain protein rich in sulfhydril and tryptophane groups (Figure 10), together with diastase-resistant PAS-positive material and various lipids, but they are neither autofluorescent nor acid-fast (53); these glands also contain esterase and a variety of other enzymes (1). The egg shell contains a modest admixture of proteins and lipids, but its main structural component is a diastase-resistant PAS-positive, refringent material that shows strong orange-yellow autofluorescence and is relatively resistant to a variety of strong and weak acids, bases, and detergents (29). The miracidial envelope contains largely of acid mucopolysaccharide. The Hoeppli phenomenon presents a combination of the histochemical affinities of egg shells and cephalic glands (Figure 11) and in this respect differs from *in vitro* circumoval precipitate, which lacks an identifiable egg shell component. In analogy with the zonation shown by immunofluorescent studies, the outer Hoeppli zone shows a strong affinity for protein rich in indole groups, consistent with the presence of host antibody. These findings suggest that antigenic secretion of the cephalic glands may escape the egg via submicroscopic pores of the egg shell and may then form an antigen-antibody complex that results in subsequent decomposition of the delicate outer layer of the shell; alternatively, the diffusible product may itself contain an enzyme that catalyzes shell decomposition (53). Since the Hoeppli phenomenon forms at the point of miracidial maturity, this product might have the role of a hatching enzyme in the natural reproductive cycle. Further studies of the antigenic components and enzymes of schistosome eggs are urgently needed.

Little is known about the catabolism of egg shells in the granuloma, which extends through its long phase of involution past the time of disappearance of diffusible antigen. Since lysozyme plays an important nonspecific role in defense against mycobacteria (40), this or similar enzymes of monocytic origin (43) may be in-

FIGURE 11. Egg of *S. mansoni* in mastomys liver, stained by the dimethylamino-benzaldehyde nitrite (Adams, 1960) method for indole groups (x 420). Both the cephalic glands and the early Hoeppli phenomenon seen along the left egg-shell border are strongly positive.

volved in schistosome egg-shell catabolism. Identification of the distinctive egg-shell material, likely to contain highly polymerized glucosamine or glycoprotein (55), could be a first step into an interesting borderland of biochemical immunology, since there is evidence that granuloma resolution can be accelerated by sensitization although egg shell material is not yet known to be responsive to any physiologically active mammalian enzymes.

Immunological significance of pseudotubercles

From the aggregate evidence presented here, pseudotubercles appear to function as auxiliary subunits of the lymphoreticular "establishment" in handling particulate pathogens from which diffusable antigen is gradually released, thus inducing antigen sequestration *in situ*. These macrophagic cell factories act as immunological receptors and effectors and potentiate the ability of the sensitized host to catabolize antigen and to break down residual inert matter during granuloma involution. In this manner, granulomas are uniquely equipped to defend the host against a variety of antigens produced by microorganisms and haptenic chemical deposits too large or too toxic to be handled by single cell units.

Askonas and Humphrey have suggested that antibody may be locally generated in adjuvant-antigen granulomas (3); since pseudotubercles show a gradient of antigen concentration that decreases toward their periphery, antibody might be a function of lympho-plasmocytic cells that surround the phagocytic core of mature granulomas. This attractive hypothesis would partly account for the enhanced antibody-generating potency of antigen-adjuvant mixtures and for the high antibody levels found in schistosomiasis. However, in order to avoid misunderstanding (12) it should be re-emphasized here that antigen sequestration cannot totally prevent diffusible antigen from reaching and activating the entire lymphoreticular system; therefore, peusdotubercles act by supplementing, rather than supplanting, the classical immunological responses to antigenic stimulation.

Summary

The immunopathology of schistosome pseudotubercles has been reviewed, with particular emphasis on antigen sequestration and on accelerated antigen destruction in sensitized hosts. The sequence of events in primary and secondary pseudotubercle formation has been analyzed and correlated with currently available data on the nature of schistosome egg antigens; the miracidial cephalic glands and the egg shell have been identified as major antigen sources. The interrelated cellular and humoral factors in host sensitization have been explored, with particular attention to precipitate formation and to central necrosis in pseudotubercles, and the Hoeppli phenomenon has been identified as an *in vivo* antigen-antibody complex. The modifications of granulomatous inflammation and its concomitant pathology in the course of natural schistosomi infection have been summarized;

…nally, a brief evaluation of the immunologic role of granulomas has been presented.*

Moderator: I have nothing to add to the excellent report presented by Dr. von Lichtenberg except to endorse his view that in chronic natural infection the host-parasite balance is the result of antagonistic forces of both the host and the parasite and not of unresponsiveness on the part of either.

It has been speculated even today during our session that well-adapted parasites are recognized by the host as "self," whereas pathogenic parasites are definitely regarded as "non-self" and therefore elicit an immune response. It should be borne in mind, however, that parasites undergo antigenic changes within the host as a result of antibody production, and that sequestered antigens may therefore be released in the course of infection. The liberation of functional antigens may lead to protection of the host, to immunity, but if the antigens released are nonfunctional the coating of the parasite by the corresponding antibody may very well protect the parasite by a mechanism similar to the enhancement phenomenon in transplantation immunology. Thus the elimination of worms could be interpreted as the result of the predominance of the stimulus provided by functional antigens over the enhancement effect induced by antibodies to nonfunctional antigens. Contrariwise, if enhancement overcomes immunity, tolerance will be established and the infective larvae will remain in a dormant state.

This last mechanism may be operative in helminthic infections terminated by self-cure, which have been observed in both natural and experimental infections. As was pointed out by Dr. Weinstein, sheep in Great Britain subject to continuous infection with larvae of *Haemonchus contortus* show an increased output of eggs every spring, and this spring rise is followed by self-cure and by a period of protection over the rest of the year. It seems well established that in this particular case self-cure is associated with the molting of dormant larvae of the fourth or fifth stages and the concomitant liberation of potent functional antigens with the excreted fluids.

I hope that Dr. Soulsby will comment later on the possibility that the trigger mechanism of self-cure in the *Haemonchus* infection may essentially be a switch from tolerance to immunity through the decoating of dormant larvae and the formation of antibody to previously masked functional antigens.

In the case of schistosomiasis, self-cure may also be achieved, for instance, in laboratory monkeys or in poor hosts such as white rats, guinea pigs and rabbits. In mice and in men, however, the infection usually leads to a chronic state, in which the parasite is sequestered and slowly destroyed in a pseudotubercle. The situation is complicated by the migration of the parasite to ectopic areas, particularly in the liver, causing widespread damage. It has even been claimed that chemotherapy of schistosomiasis in heavily infected mice aggravates the disease as a result of the arrest of dead schistosomes and the development of obstructing lesions, which leads to a distortion of the intrahepatic vascular system and to the so-called axial fibrosis as it is seen in human liver preceding the coarse fibrosis (Symmer's clay-pipestem cirrhosis) characteristic of the later stages of the disease.

There are indications, however, that this potential danger of chemotherapy has been exaggerated. Moreover, although lesions may appear surrounding dead parasites, schistosome or adult worms, the basic histopathological damage in *Schistosoma mansoni* seems to be a granulomatous obliterating vasculitis produced by embolized eggs. It is also believed that living eggs in the tissues may be necessary for the development of immunity in the mouse. If the miracidium is an important source of functional

* The tragic death, in 1965, of my collaborator and friend Ramón Gómez Mazzei has been a permanent loss to science and to humanity. Many others have given aid and encouragement to this work, which now extends over more than nine years, and as far as possible their share has been acknowledged in each personal article reviewed as a source. Whatever new understanding may have emerged from these studies, let it be a tribute to the memory of Ramón and a source of satisfaction to all who have so generously helped me in this labor.

antigen, the granulomatous reaction around the egg could be compared to immunization with antigen and Freund's adjuvant, and this could lead either to the formation of antibodies or to the development of delayed hypersensitivity.

Suggestive evidence has been presented in favor of an immunological barrier to the penetration of cercariae of S. *mansoni* in rats, when applied to skin sites previously infiltrated with reagin-like homologous PCA antibodies. However, this barrier seems to be effective only in the rat, which is endowed with a conspicuous degree of natural resistance. No protective effect was obtained, so far as I know, under similar experimental conditions with reagin-like antibodies from superinfected monkeys when cercariae of both *mansoni* and *japonicum* were applied to skin treated sites.

These are only a few reflections I should like to submit for your consideration in introducing the discussion of the topic presented by Dr. von Lichtenberg.

Dubos: When I spoke a little earlier I apparently gave some of the participants the impression that I was downgrading the importance of specific reactions in immunity and allergic processes. This was very far from my thought. In fact, it is because I wanted to make it clear that I considered the specific phase of the immunological process fundamental to all phenomena of immunity that I introduced my remarks with a statement that I was going to speak only of the second part of Dr. Biro's scheme.

So let me just restate my personal views on the mechanisms through which specific immunity processes are converted into reactions that are effective in controlling infection. There is no doubt whatever in my mind that the first and fundamental aspect of these processes is the classical induction of immunological response, with all its specificity and occurring in different forms with which we are familiar, including the production of the classical antibodies and the production of the complex set of reactions that result in delayed hypersensitivity.

But it is also my conviction that after this mechanism has been triggered into activity, there follows a set of secondary reactions that are of immense and probably decisive importance in determining the manifestations of the specific immunological process.

Since this aspect of the nonspecific consequences of specific immunological processes has not been well studied, I can at best give a few suggestions on how it might affect the fate of the infection. So please consider the two or three (or perhaps four, if they come to my mind fast enough) mechanisms that I am going to suggest not as exhausting the possibilities but merely as illustrations.

To begin with, it has been demonstrated time and time again that antigen-antibody reaction does result in the activation of several enzymes, in particular of proteolytic enzymes. All of us are familiar with the classical demonstration that cathepsins can attack certain parasites, certain worms especially, and it might well be imagined that the activation of a proteolytic system through an antigen-antibody reaction might create active enzyme mechanisms that can attack the parasite.

It has also been demonstrated countless times that an inflammatory reaction is accompanied by a greater acidity of the site of the inflammatory reaction through an exaggeration of the glycolytic processes. As you well know, measurements of local pH have shown that the pH at the site of the inflammatory reaction can fall to 4.5 to 5. There is very little doubt that this change of pH can affect certain parasites directly, and perhaps indirectly, by providing an environment in which certain enzymes can act.

It has also been shown more recently that lysosomes and all sorts of granules will be caused to disrupt and discharge their contents as a result of contact between a certain sensitized cell and the antibody capable of reacting with that cell. Here again the potentialities of release of biologically active substances are enormous. You are probably familiar with the recent demonstration that probably all the lysozyme is stored in lysosomes and is released only when the lysosome is destroyed. You may also

be familiar with the release of those small basic peptides of various kinds that have been shown to be stored in the lysosomes and to be released and to adsorb nonspecifically on the surface of many types of parasites.

I believe I could spend the next fifteen minutes listing from memory some of the biologically active substances that are either released or rendered active by different forms of immunological reactions, but I wish only to conclude by summarizing in one phrase the overall point of view that I have tried to communicate to you: All immune processes involve at the beginning some highly specific reactions. They are those of classical immunological specificity. But once this specific triggering of the reaction has occurred, then there is a nonspecific release of biologically active mechanisms that certainly operate in all pathological processes.

Smithers: I should like to take up a couple of the points made by the Moderator. First, the suggestion that the egg and the granuloma formation may be responsible for inducing the protective response in schistosomiasis. After the last couple of years' work in our laboratory, I am absolutely convinced that the egg has nothing at all to do with the development of protection against a challenge infection. The evidence for this comes from some worm transfer experiments we have been doing—transferring adult worms from hosts into normal rhesus monkeys.

Moderator: Excuse me, Dr. Smithers. I did not mean to suggest that. I compared the miracidium-releasing antigen in the living egg to an immunization with Freund's adjuvant, but I did not mean that this was a protecting antibody. It could lead to delayed hypersensitivity or to antibody formation, but I did not mean to say that it was protective.

Dr. Smithers: I am sorry; I must have misunderstood you. But to finish (because I believe it is important that this point should be made clear), protection is induced by transferring male worms alone, or female worms alone, or even worms that have been cut in half and are still alive although they produce no eggs; but protection cannot be induced by introducing into the hepatic portal system of a normal monkey half a million living viable eggs. This has no effect upon protection at all. Although, as Dr. von Lichtenberg has shown, the egg is responsible for reducing the granuloma in sensitized animals, it has no effect on the protection against a challenge.

The other very interesting point was the fact that worms may become coated by antibody, and in this way they may protect themselves against the host's immune response. In our experiments we have transferred worms from hamsters and mice and monkeys into normal monkeys. We find that the results are different, depending on the donor animals used. If worms are transferred from monkeys to monkeys, then the transfer take is very good, and after a week or so eggs begin to appear in the recipient, and so on. If worms are transferred from hamsters to monkeys, then the results are extremely poor. Very few eggs are produced, and the worms are quickly killed off. If worms are transferred from mice into monkeys, you get an in-between stage. The initial response—that is, the egg production—is poor, but after five or six weeks the egg production increases until a level is reached that is comparable to that of monkey-to-monkey transfers. We believe this shows that the worms have become adapted in some way to their definitive host; thus, worms from hamsters are adapted to hamsters and cannot make the necessary readaptation when they are transferred to the monkey host. It could well be that the worm has become coated with hamster antibody; when we transfer this worm to the monkey, the monkey does not recognize it as a schistosome worm but recognizes it as a hamster, and consequently kills it off. However, this is pure speculation.

Cohen: It does not seem appropriate for a biochemist to discourage people from recognizing the importance of the biology of a cell, because I have participated, as so many of you have, in grinding things up and looking for this or that. But I should like to mention an observation—one I am not sure has been published yet—that bears in some rather important

respects on some of the discussions today. This observation, reported by Dr. Jerry Gross of the Massachusetts General Hospital, has to do with the disappearance of the tail of the tadpole under certain conditions. A rather rapid and extensive dissolution of collagen, which exists in rather large quantities, is involved. As biochemists are inclined to do, Dr. Gross made a very serious effort to look for the enzyme collagenase in the system thus triggered. It seems very clear that in this system there is neither a proenzyme nor an inhibitor that normally represses the enzyme. But apparently there is a layer of cells in the tail (and I should interject here that if the tail is cut off and put in a Petrie dish under sterile conditions with thyroxine, the same process takes place as in the whole animal) that is separated from the collagen fibers, and when certain changes occur and these cells come into contact with certain segments of the fibers, and only under these conditions, a specific collagenase is induced in the cells, and then the dissolution takes place.

This leads me to suggest that the absence of an enzyme may be very important. This is not a lysosome process; I do not quite share your enthusiasm about the lysosomes. I think it is another kind of process. But it seems to me to point to the fact that a cell, whether a parasitic form or a host cell, may have potentials because of the presence, if not the actual adherence, of specific proteins or other cells that lead to the induction of activities not found in the absence of those particular circumstances.

I believe Dr. Gross showed that the effect was puromycin-sensitive, and thus truly an induction. In any case, what this means is that, biochemically, the biology of the cell is a challenge. Simply grinding up structures, as one of the earlier speakers said, shows nothing; one does not know for sure what it is one is extracting, nor, afterward, what one has extracted. Other dimensions may have a bearing. I think this is an excellent example of a specific effect induced to serve a particular role—in this case, to dissolve the collagen—and the enzyme is not present or not discernible under any circumstances until a certain biological event occurs.

Lichtenberg: I want to ask Dr. Soulsby, since his findings have had such a vivid response here, for some additional comment about the significance of what happens to the third-stage larvae specifically in ascariasis; whether he believes that something like a parasitical effect could occur simply by obstructing the transit of an organism that is in the process of migration and maintaining it at a stage that is not the one corresponding to its subsequent metabolic requirements. Is this part of his interpretation of the cell adherence phenomenon, and is it something that might happen in other helminthic diseases in general?

Soulsby: I should like to think that these cells are so responsive that on a second infection they would behave as you suggest. I am not sure, though, that this is the case. In examining an infection serially, it always seems that the cellular aspect of the response is a little behind the progress of the parasitic infection. On second infection, it appears to catch up. I am not sure what delays the infection so that the cells can catch up; it may be antibody. The sequence may well be that antibody slows down an infection and then the cells have a chance to operate.

Remington: I hesitate to raise another controversy here, and this is not necessarily for the record. As I have sat here today I have wondered about something that I think may also be in the minds of many of you. Are we sitting here in a relative vacuum? Are we, each of us, traveling down different paths in our own research toward similar goals, and are all these paths going to end in different places? Cannot there somehow be more of a cohesiveness—a recognized "core" for each problem and some sort of cooperative effort to answer the "core" questions? As a physician interested in clinical infectious diseases, I am interested in host response and immunity to infectious agents, and in the prevention of infections and the morbidity and mortality caused by them. The virologists and bacteriologists now have a large armamentarium of vaccines and effective chemotherapeutic agents with which to prevent, treat, and/or study their infectious diseases. And

how stands the parasitologist in comparison? More infection and disease than can be claimed by either of the other groups, with relatively little in the way of advances to compare with theirs. Is this because there is a lack of knowledge of how to approach parasitic diseases, a lack of knowledge of the parasite and its host's response, or is the lack also one of no common path or goal, or are these simply ill-defined? Is it the diffuseness of efforts that is causing this scientific lag?

We have seen today that there seems no shortage in talent—veterinary medicine, pathology, immunology, parasitology. But there seems to be a very real gap in the sharing of knowledge—not in willingness to share but perhaps in facilitation. I wonder what is going to be done about this. How many more years are we going to remain in this relative void without significant advances toward answering age-old problems? Are we to be satisfied with advances that carry us only micrometers toward answering a core question, or shall we seek ways that may help us leap by giant steps toward answering questions that plague those concerned with the health of the world? It may be that the answer is greater cooperation among us all.

Moderator: I think that we are making that sort of effort here today. How far we will succeed, I do not know. But we are making an effort to put basic immunology and immunoparasitology together.

Bruce-Chwatt: As a malariologist of long standing, I feel much more optimistic than you seem to be. I feel that a very great deal has been done with regard to the immunological approach—not only toward an understanding of the process of infection and response to infection, but also toward coming to grips with the problem of the still hypothetical malaria vaccine. I feel that we understand today better than ever before the interrelation between the humoral and the cellular aspects of immunity in malaria. We are still very far indeed from the end; for a long time we are going to have to rely on chemotherapy of malaria. Nevertheless, I feel that we are much closer to understanding and solving the problem of malaria.

Let me put one question here to my friend and colleague Dr. Brown, who has described so well his work on the antigenic change in malaria parasite in the course of infection. Does he believe that this undoubtedly important phenomenon he has discovered will have an impact—negative or positive, probably the latter—on the development of the still hypothetical and faraway malaria vaccine?

Brown: No, I do not believe it will affect the ultimate development of the vaccine. I think that, unfortunately, antigenic variation can be used to explain anything—especially any negative result. If you do not happen to protect by immunization one time, you say you have used the wrong variant, or something of that sort. But in fact, to speak in particular of Freund's original experiments, he kept his strains under quinine and they were obviously relapsing continuously. Almost certainly the variants he challenged with were different from the ones that he immunized with. I think there is good reason to suppose that with suitable adjuvant it should be possible to immunize quite effectively with the blood forms.

Bruce-Chwatt: I forgot to say before that a great deal of what we know today about the immunology of malaria is due to the tremendous efforts of Dr. Sadun and his group, and I should like here to pay tribute to his work.

Cohen: I hope that Dr. Remington will not think I am directing this remark at him, but I think what we have seen today is an example of the fact that you cannot parlay two half truths into a whole truth. Until we have whole truths to deal with, we are going to be a long way from dealing as directly with the situation as he believes we must. I am not against getting together, but we need more information, and information substantial enough to mean something, before we can make much of it.

Remington: In essence, I certainly agree with Dr. Cohen. However, we are well aware of the government-funded chemotherapy programs for malaria in the last world war and for

cancer at present. Most of us in basic research are loath to perform directed research or even to take contracts that dictate a complete program differing from our major interests. There is evidence in favor of *laissez-faire* for scientists and their research. But there is also evidence in favor of directed programs formulated by experts in the field who consider that certain experimental designs will lead to more rapid answers to questions vital to world health. I want to proceed in my work without interference, and I hope that government granting agencies will be tolerant of my approach and views. Yet I also consider it very important that someone, somewhere, be able to ask the "core" questions on each of the maladies of mankind and that there be institutions that will help guide research more directly to the answers, by a less devious route than is being taken in many laboratories today. To put it in the vernacular, "someone must keep his eye on the ball." The "ball" that is rolling here today is the parasitic infestation of millions of people. Public health preventive measures, including vaccines or specific drug therapy, must be found for each of these infectious diseases. My answer to the malariologists is that I sympathize with the splendid statement made by Martin Young several years ago in a lead article in the *Journal of Tropical Medicine and Hygiene:* "Medical progress must allow for the eradication of the disease before liquidating the investigator." In this country the malariologist was "destroyed" before malaria itself was eradicated. Now, confronted with the problem of malaria in Vietnam, we have a resurgence of malaria research—because someone, somewhere, has his "eye on the ball" and is making funds available for research. I hope that we can do the same in other fields of parasitology.

Waterlow: As a Committee member who knows virtually nothing about immunology, I have the impression at the end of today that, left to themselves, the body's defenses against these parasites are not very good and need supplementing—just as I think that with many forms of bacterial disease most of us would take antibiotics or sulfonimides and not rely entirely on the antibody response. The impression I have received is that possibly the point to which most attention needs to be paid, from a practical point of view, is the biochemistry of the parasites and the point at which they can be hit hard with drugs without hitting the host.

Moderator: Gentlemen, I am afraid that we must bring this to an end. Dr. Kagan has been kind enough to take on the task of briefly summarizing today's discussions, since Dr. Oliver-González was unable to attend.

Kagan: Mr. Moderator, I feel like the matador who comes into the arena after the bull is dead. It has seemed to me, in thinking about what to say in summing up this very interesting day, that most of my ideas have already been expressed within the past half hour. I should like to point out, as a public health parasitologist, that the comments made by Dr. Remington were uppermost in my mind. We have to remember that we are talking about diseases that are of tremendous importance in the world, and our ultimate aim is to provide tools and measures for their control and possible eradication.

We are never going to arrive at a stage at which we can control and perhaps eradicate parasitic diseases unless we have the whole truths that Dr. Cohen alluded to. It is only by wedding basic research to applied research that we can reach that stage.

I was also impressed by Dr. Dubos' remarks. It occurred to me that fifty years ago people said in print that parasitic diseases did not invoke an immune response. Dr. Taliaferro was one of the early investigators who pointed out repeatedly in his writings the similarity between the immune response of microorganisms and that of parasitic organisms. Today we have been reminded again that all the mechanisms that hold true in immunologic problems related to viruses and bacteria are also true of parasitic organisms, and that some of the advances made in those fields can be exploited in working out the problems in parasitology.

We are relative beginners in the immunology of parasitic infections in terms of working out mechanisms. We are recovering from the flush

of some initial successes in this area to realize that we cannot look for a primary mechanism in the immunologic response of parasites, that they are exceedingly complex, and although we may not be able to lump immunologic mechanisms, we can lump concepts. The more we study the response of the host to his parasite, the more complex it becomes. Dr. Smithers talks about the schistosome egg not being the immunologic stimulus in the monkey. This may be true for the monkey, but perhaps not for another host. The recent work of Lichtenberg and Sadun in a detailed study of the immunologic response to schistosomes in a variety of hosts indicates the complexity of response on both the cellular and the humoral level.

Another point I want to make is that before we arrive at "whole truths" we first have to "survey the territory" we are studying. We have a big field with many organisms, many species. Characterizing and describing the immunologic responses and developing assay methods for their study have occupied parasitologists for the past twenty to twenty-five years. It is only after we have such assay systems that we can begin to look for the immunologic mechanisms involved. When we have the methods we need, we can get down to some basic immunology.

Before I make some specific comments about areas that remain to be studied, I should like to revert to Dr. Cohen's remark that we ought to characterize some of these antigens. We have not done so because it is difficult work, and until we have identified specific antigens and characterized their biological and immunological role, this aspect of research will lag. After this has been done, I believe, we can devote the energy necessary to characterize the antigens, identify their subunits, and perhaps even attempt to synthesize them and attach them to artificial groups to make vaccines. But we are a long way from that, because we have not done our homework in all these areas.

The hallmark of this meeting, I think, has been complexity—complexity of antigenic structure in malaria and other microorganisms outlined by Dr. Brown, complexity in pathogenic structure as outlined in my presentation. Dr. Goodman brought up the question of the complexity of immunoglobulin and alluded to the complexity that goes beyond the IgM, IgA, and IgG level. We now have IgD and IgE. But even just in the IgG class we have at least four subgroups that have light chains. We know that the light chains have at least ten subgroups. We know we have kappa and lambda light chains. We thus have a whole host of complex immunoglobulins and we have no idea of their biological function. Not only that, we have no way of studying them adequately as they relate to parasitic diseases and infections.

A tremendous challenge confronts us, therefore—on one hand to elucidate the antigenic structure on one side, and on the other to relate these to the development of specific antibodies in the various subclasses by other immunologists and by parasitic immunologists.

In conclusion, let me point out that we are not going to find our answers in studies of the humoral aspects of parasitology, and we are not going to find them in studies on the cellular aspects and on delayed hypersensitivity. We shall find the answer in a combination of the two and in the interaction of antigen-antibody complexes on these cellular reactions.

I believe that parasitic organisms use every immunologic trick that has been developed by a living organism. We know some of them. There are immunologic processes that we are just beginning to become aware of. As we understand how they relate to other microorganisms, we shall find that these processes can be applied to the study of parasitism of helminths and protozoa. In this lies the challenge and the need for people with diverse backgrounds to apply their knowledge to the study of parasitic infection. Only by wedding basic immunologic information to practical goals will we ever reach a point at which we can diagnose parasitic infections with confidence, prepare vaccines for their elimination and for host protection, and develop control programs in the world.

Moderator: I close this meeting with the

feeling that we have indeed accomplished our objective, which was to encourage new approaches to the problem of parasitic immunity by utilizing concepts and methods of modern basic immunology. On behalf of my colleagues of the Advisory Committee, I wish to express our gratitude to each of the speakers and also to Dr. Martins da Silva and to Dr. Louis Olivier, who are the persons chiefly responsible for the organization and plan of this session.

Appendix

IMMUNOLOGIC ASPECTS OF PARASITIC INFECTIONS: SUMMARY*

Jose Oliver-González

Past accomplishments on immunologic aspects of parasitic infections were very well reviewed by a pioneer worker and authority in this field, Dr. William H. Taliaferro. Among the most significant of these accomplishments have been the recognition of host reaction against parasites mediated through specific antibodies and cellular by-reactions, and of the important part played by lymphocytes—migrating to foci where parasites are present and changing into macrophages—in immune reactions. The biochemistry of the parasites and antigenic constituents have been also emphasized, mostly in connection with serological diagnosis. The electron microscope, isotopic markers, and autoradiography could be used to great advantage. Electron microscope studies on the fine structure of parasitic tissue are revealing important features of the secretion of antigens.

Brown and other investigators mentioned fundamental points in connection with the nature and variation of parasitic antigens. The antigenic structure of parasites, particularly protozoa, changes during infection; with such versatility, they become less vulnerable to host immune reactions. These changes, however, need to be correlated with changes in enzyme characteristics and followed by tissue culture studies.

The antigens responsible for immunizing the host do not originate only in the parasite but also consist of host-parasite complexes. Antibodies that develop against these host-parasite complexes may lead to phenomena related to autoimmune diseases.

A great deal of progress has been made in the immunodiagnosis of parasitic infections, although more specific antigens are needed to increase the efficiency of the diagnostic tests. Very few antigenic substances of established purity have been isolated. As Kagan emphasized, immunochemical analysis of the antigenic mosaic will lead to effective methods for the assay of purity.

High immunoglobulin levels are encountered in parasitic infections. Hypergammaglobulinemia occurs in schistosomiasis, malaria, trypanosomiasis, and Chagas' disease, at different times releated to course of infection. An increase in immunoglobulin is not necessarily due to an increase in specific antiparasite antibody.

Reagins have been demonstrated during helminth infections of man and other hosts. These are induced by the living organism and not by dead worm materials. Their role in protective immunity is now being uncovered.

Parasitologists are still faced with the problems of understanding the role of antibodies in protection against infection and of preparing effective vaccines. Very little is known about the characteristics of antibodies formed in response to parasitic infections; this knowledge is necessary for additional light on the mechanisms of parasitism and host response. New techniques have been developed for characterizing these antibodies. By means of exclusion

* Dr. Oliver González, who was unable to attend the Special Session, prepared this paper after reviewing the summaries of the papers presented.

chromatography on columns of cross-linked dextran gels, the molecular weights of unknown proteins may be determined through correlation with the elution of proteins with known molecular weights. Demonstration of IgM and IgG antibody during different stages of infection has proved to be of diagnostic significance in certain parasitic infections.

The role of the lymphocyte and macrophage in immune reactions is again emphasized. Immunity has been transferred by lymph nodes. The role played by lymphocytes in delayed hypersensitivity has been found to be of importance in resistance. The role of the macrophage in immunity is not clear, since in some infections parasites multiply within the macrophage whereas in others intense phagocytosis of the parasite is a manifestation of acquired immunity.

Weinstein summarized very effectively the observed effects of immune response on parasites. These effects, at a gross level, include such phenomena as retardation of movement, immobilization, and obliteration of orifices and lumena. The effects at physiological and biochemical levels are a decrease in infectivity, stunting, changes in uptake of dyes, and alterations in metabolic processes.

Lichtenberg used infection with *Schistosoma mansoni* to illustrate host reactions against parasites. The antigen proceeding from the schistosome egg is withheld or "sequestered" within the pseudotubercule. With sensitization of the host, cell reaction and antigen destruction are greatly enhanced. The antigen-antibody precipitate has been shown *in vivo* around eggs in animals highly sensitized (Hoeppli's phenomenon).

Comments

Although the need for specific antigens is very obvious, recent investigations indicate that parasitic species, particularly nematodes, contain common antigens that may be responsible for a particular reaction or pathological phenomenon within the host. A glycoprotein has been isolated from the coelomic fluid of adult *Ascaris suum* that has been detected in the larvae of *Trichinella spiralis*, in third- and fourth-stage larvae of *Ascaris lumbricoides* and *Toxocara canis*, and in adult *Dirofilaria immitis* (1). Sera from children with the syndrome of visceral larva migrans reacted positively against this antigen in direct and indirect hemagglutination tests. This suggests that the antigen responsible for the syndrome is present in a large number of parasites.

The presence of specific antigens in developmental stages of parasites is still debatable. Some investigtaors claim (2) that stage specificity may be only apparent, since immunizing extracts from different stages may stimulate demonstrably distinct antibodies by virtue of the differences in their relative proportions of common antigens. Stage specificity may also be a function of the time at which serum has been obtained. The presence or absence of stage-specific antigens should be studied in relation to enzyme systems possessed by the various forms, since enzymes probably represent these specific antigens.

Immunization against infection with antigen-antibody complexes, rather than with antigen alone, has been overlooked. Antigen-antibody precipitates have been shown to occur *in vivo;* therefore, this complex must play a role in host-parasite immune relationship.

Investigations on immunity to parasitic infections are burgeoning as a result of new techniques, the advance of knowledge about antibody proteins, and newer methods for isolation of antigens.

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JOSE OLIVER-GONZALEZ: Summary

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