BIOCHEMICAL IDENTIFICATION OF THE LEISHMANIAS[1]

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Isozyme electrophoresis provides a useful tool for comparing diverse populations of leishmanial parasites. The account presented here describes the basic steps involved and the results obtained by various research projects making use of this procedure.

Introduction

The taxonomy of the leishmanias is based on an intricate combination of features that includes "extrinsic" epidemiologic and biologic characters—such as the clinical picture of the infection in man *(1, 2)*, experimental and natural development of the infection in sandflies, behavior of the organism in hamsters, growth rates of the organism *in vitro*, and morphology—supported by "intrinsic" characters such as serotype, isozyme patterns, and DNA analyses *(3, 2)*.

Within this framework, undue emphasis on clinical presentation may lead to confusion. At least two etiologic agents can cause visceral leishmaniasis in man *(4, 5)*. Walton and Valverde *(6)* have suggested that race influences the development of mucocutaneous disease, and it is now well-known that there is a genetic basis for susceptibility in certain laboratory hosts *(7, 8)*.

In the most recent classification *(9)*, the *Leishmania* genus is divided into three sections on the basis of the site of development of the infection in sandflies. These sections are: the Hypopylaria, comprised of two Old World lizard species that develop infections in the posterior intestines of sandflies; the Peripylaria, comprised of two Old World lizard species and the New World *Leishmania braziliensis* complex, all of which develop infections in both the anterior and posterior intestines of sandflies; and the Suprapylaria, comprised of the New World *L. mexicana* and *L. hertigi* complexes, the New and Old World *L. donovani* complex, and the Old World *L. tropica* and *L. major* complexes, all of which develop infections in the anterior intestines of sandflies. This classification system is analogous to that used for mammalian trypanosomes *(10)*, where development in the vector is considered a sound indicator of major biological differences.

The correct section classification is not always clear, however, if the determination is based on experimental sandfly infections *(2, 11)*. Therefore, an evident need exists to exploit further parameters for taxonomic purposes—parameters that do not depend on complex interactions between the parasite and a vector or mammalian host. This is particularly true in cases where correct medical or epidemiologic identification of the parasite is the goal. It is thus significant that such identification can theoretically be attained by examining selected features such as isozyme profiles or responses to monoclonal antibody reagents *(12, 13, 14)*.

Of the diverse range of biochemical procedures used to help distinguish leishmanias, the relatively simple procedure of isozyme electrophoresis is the most widely applied. This method has made major contributions to the understanding of the epidemiologies of cutaneous and visceral leishmaniasis. Partly for these reasons, most of the review presented here is devoted to consideration of the identification of leishmanias using isozymes.

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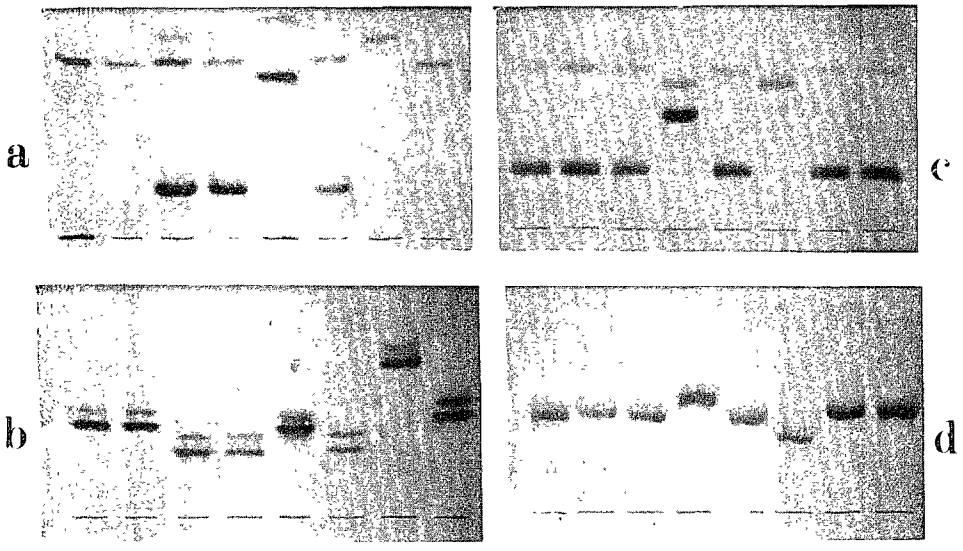
The Principles of Isozyme Electrophoresis

The theoretical basis of isozyme electrophoresis is very simple: Crude, soluble extracts of organisms are prepared by freeze-thawing or sonication and subsequent centrifugation. These extracts are applied to an electrophoretic plate; and, in response to a molecular surface charge, the extract's components move toward the anode or cathode at varying speeds. After electrophoresis, bands of a selected enzyme can be detected by overlaying the plate with a specific substrate. The action of the enzyme on the substrate produces a specific product. The product is linked to one of various staining procedures, some dependent on fluorescence. Differences in isozyme profiles suggest differences in the genes controlling production of the enzymes involved, and hence imply genetic differences in the populations of organisms under study. If a sufficient number of enzymes are used, the absence of such differences implies that the populations, while not necessarily identical, are closely related. However, careful standards of comparison are required in order to exclude complicating secondary factors *(15)*.

Polyacrylamide gel, starch-gel, and cellulose acetate have been used as matrices for isozyme electrophoresis. Each has corresponding advantages and disadvantages *(16)*. Until recently, starch-gel was the most favored. However, at present the simplicity of improved cellulose acetate membranes is proving attractive. The separation in starch depends upon both a sieving



Photographs of routine starch-gel electrophoretic plates showing enzyme differences between various *Leishmania* species and subspecies. The enzymes shown are: a (top left) nucleosidase (E.C.3.2.2.1, NH), b (bottom left) glucosephosphate isomerase (E.C.5.3.1.9, GPI), c (top right) NH, and d (bottom right) 6-phosphogluconate dehydrogenase (E.C.1.1.1.44, 6PGD). The *Leishmania* species and subspecies tested in a and b (from left to right) are: 1 and 2 *L. mexicana amazonensis*, 3 and 4 *L. mexicana mexicana*, 5 *L. m. aristedesi*, 6 *L. m. pifanoi*, 7 *L. enriettii*, 8 *L. m. garnhami;* and the subspecies tested in c and d (from left to right) are: 1, 2, and 3 *L. braziliensis braziliensis*, 4 *L. b. guyanensis*, 5 *L. b. braziliensis*, 6 *L. b. panamensis*, 7 *L. b. braziliensis*, 8 *L. b. braziliensis*. The scale is indicated by the threads at the origins, which are approximately 1 cm long. Courtesy of David A. Evans and Valerie Smith; NH procedure modified by S. M. Lanham and C. I. Baldwin (in preparation).

effect and molecular charge differences, while separation in cellulose acetate is charge-dependent only. Comparisons of the two techniques show that ideally both should be used, since resolution of some enzymes is best accomplished with starch and resolution of others is best done with cellulose acetate *(17,* Lanham *et al.,* in preparation). Isoelectric focusing (IEF) in polyacrylamide or agarose matrices (with pH gradients established by ampholines) enhances resolution, so that more isozymes may be revealed; but due to higher cost and more demanding technical requirements, IEF is not yet suitable for extensive epidemiologic studies.

Stocks and Strains

A series of terms has been defined *(18)* to standardize presentation in the literature and enhance communication between research groups. In our work on isozymes of leishmanias, we have employed the terms "stock" and "strain" in the manner they have been defined for work with trypanosomes, namely:

- *Stock*—the population derived by serial passage *in vivo* and *in vitro* from a primary isolate; there is no implication that such a population has been characterized or is homogeneous.
- *Strain*—a set of populations originating from a group of organisms of a given species or subspecies present at a given time in a given host or culture and defined by the possession of one or more designated characters.

Another term, "zymodeme," has been used for trypanosomes but has not been widely applied to leishmanias. A zymodeme is defined as populations of microorganisms that differ from others of the same species or subspecies in a specified enzyme property or set of enzyme properties.

In the case of *Trypanosoma cruzi,* zymodemes have been designated by radical dissimilarities in sets of isozyme properties rather than in single isozyme properties. In the case of African trypanosomes, however, the term zymodeme has been applied to trypanosome stocks with any single unique isozyme character. As there is now evidence that the enormous variation in isozyme patterns of some African trypanosomes is generated by random mating *(19),* zymodemes defined by single isozyme characters may correspond to the level of individual variation.

With regard to leishmanias, it has not yet been proven that the leishmanias are altogether asexual organisms. The term zymodeme, if applied to leishmanias, should therefore be employed with caution, bearing in mind the need for clarification of its usage.

Isolation and Maintenance of Leishmanias

Only prolific *Leishmania* infections in experimental animals, such as *Leishmania mexicana amazonensis* infections in hamsters, can provide adequate material for isozyme studies directly. Therefore, organisms for isozyme characterization are usually harvested from large-volume cultures supporting rapid and abundant growth. Theoretically, culture media can also be used for the isolation of leishmanias from all sources, as well as for obtaining the material necessary to perform electrophoresis. The ideal all-purpose culture medium should be a lyophilizable liquid that is sensitive to low inocula, protected against bacterial and fungal contaminants, inexpensive, having long shelf-life, and suitable for direct use in field situations by "through-the-cap" inoculation *(20).* In practice, the ideal medium has yet to be found; and, because of the extraordinary diversity of leishmanial parasites, it is unlikely that any one medium will satisfy all needs.

Insect culture media might be expected to readily support the growth of leishmanial parasites; and Schneider's *Drosophila* medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), is useful as both a diagnostic tool and for the bulk growth of organisms. It is very expensive, however, and its sensitivity seems to be partially dependent on ill-defined qualities of FCS batches. A simple, sensitive, and widely used biphasic medium is the "Difco" medium with a distilled water overlay *(21).*

Bacterial contamination can be reduced by

the use of gentamycin (which is preferable to penicillin and streptomycin) or other broad-spectrum, stable antibiotics. The drug 5-fluorocytosine, which is converted to 5-fluorouracil by cytosine deaminase in fungi, and which disturbs protein synthesis by replacing uracil in RNA, is a useful tool for controlling fungal contamination, but it is antagonized by peptones.

Problems of contamination when culturing from open lesions, the skin of reservoir hosts, or sandfly vectors may be severe, and stringent procedures are required to exclude bacteria and fungi. Nevertheless, Arias *et al.* (unpublished) routinely culture flagellates from naturally infected sandflies. Our own experience in the inoculation of media with trypanosomes from naturally infected triatomine bugs demonstrates that cultures from the most precarious of sources can be successful. Basic portable laboratory equipment enables such cultures to be made in field localities. Whether isolates are obtained by means of animal inoculation or culture, both techniques are likely to be highly selective; that is, some organisms will fail to develop entirely, and others may be masked by overgrowth.

Cloning

Mixed infections of mammals or vectors confuse the interpretation of isozyme data. It is thus especially desirable that standard, representative *Leishmania* stocks be cloned for use in making broad isozyme comparisons. A cloning procedure used successfully with *T. cruzi* stocks involves the examination of capillary microdrops sandwiched between sterile overslips, and then the transfer of drops containing single organisms to new cultures.

The whole procedure is performed aseptically in a humidified glove-box containing a microscope. Cultures are used that contain free-swimming organisms rather than amastigotes. Microscopic examination of the drops must be rigorous, and at least an equal number of drops thought to contain no organisms must be cultured as controls. We have been unable to undertake the cloning of *Leishmania* stocks ourselves, but we believe that this procedure should be applicable. Alternatively, cloning may be performed by repeated serial dilutions. In addition, plating onto solid media and selection of individual colonies is feasible but less reliable.

Avoiding Confusion

The advent of a relatively simple and informative procedure such as isozyme electrophoresis encourages the description of isozyme profiles for leishmanias of doubtful origin and identity, sometimes with confusing results. The identity of some laboratory strains assigned to particular taxa is, in my opinion, questionable; and this identity problem may explain conflicting biological and biochemical observations in the literature.

It is worth noting, as examples of the kinds of errors occurring, that stocks received from leading laboratories for isozyme characterization and designated as *"T. cruzi," "T. rangeli,"* and *"L. braziliensis guyanensis,"* have respectively proved to be a *Leishmania* species, mixed *T. cruzi* and *T. rangeli*, and *L. m. amazonensis*. As a result, our isozyme studies have been largely devoted to newly isolated stocks with known histories. To counteract confusion, which is to be expected occasionally in any laboratory handling large numbers of organisms, we routinely question all stated labels and origins on stocks received from elsewhere, cryopreserve stocks as soon as possible, keep precise records of every laboratory procedure, and prepare Giemsa-stained thin films of all stocks when they are harvested. In addition, we believe it clearly advisable to perform all electrophoretic studies with several enzymes, under controlled conditions and using consistent standard markers. In sum, presumptive conclusions drawn from small numbers of poorly characterized stocks can be misleading.

Epidemiologic Studies

In view of the complexity of the epidemiologies of the leishmaniases—with several different or-

ganisms occurring together in small localities *(22)*—we recommend that investigations based on isozymes should initially be performed in depth on a regional basis and should address specific, relevant epidemiologic questions. Continuity with future studies can be maintained by using representative *Leishmania* stocks throughout the comparisons. Such stocks will eventually provide a means of comparing isolates from widely separated geographic areas. It should be kept in mind, however, that lack of evident difference between a wide range of enzymes from two organisms is merely a good indication of close similarity—one that may obscure minor differences involving markers that could be of vital epidemiologic or clinical importance.

Data Analysis

For making simple comparisons, enzyme profiles can be compared visually, patterns can be placed into homogeneous groups, and the dissimilarity of the groups can be roughly assessed in terms of the number of enzymes that distinguish them. If many enzymes separate two populations of organisms, the populations may be considered radically dissimilar—as in the case of *L. m. amazonensis* and *L. b. guyanensis (23)*, for example; and if few enzymes separate them—as in the case of *L. b. guyanensis* and *L. b. panamensis (23)*—they may be considered closely related. The distances that enzyme bands move away from the origin, relative to the migration of a standard colored reagent (RF values), are sometimes used to facilitate comparisons between different electrophoretic plates. The only really satisfactory way of performing comparisons, however, is to ultimately include representatives of each pattern on the same electrophoretic plate.

If a large number of stocks with complex electrophoretic patterns are to be compared, mathematical procedures may be applied. In hierarchic clustering, similarity indices are employed to express the relationships between stocks. Whole patterns or single bands can be used as characters in such comparisons. The use of single bands as characters may be more informative, but distortions will result if heterozygosity is a feature of the populations under study (see below). Similarity indices may be transformed into dendrograms, from which similarities between stocks can be readily assimilated, and delimitations of groups may become apparent. Some distortion is inevitable in the construction of such dendrograms *(24, 25, 26)*.

The ability to define groups of stocks can also be greatly enhanced by more complex means of analysis involving ordination—for example by principal components analysis. Distinct clusters may become apparent that relate to geographic, ecologic, or other epidemiologic features. These more sophisticated mathematical approaches require computer processing.

If genetic interpretation of the observed enzyme bands is possible, bands may be assigned to particular genetic loci, and allozyme frequencies can then be used to calculate genetic distances. While this approach is usually applied to sexual organisms, it can also be used with organisms that are presumed to be asexual. The larger the number of loci, the more satisfactory this form of analysis. For heterozygous patterns of enzymes considered to be multimeric, the number of bands observed exceeds the number of alleles. Bands resulting from heterogeneous subunit combinations are ignored. Genetic distances may also be expressed in the form of dendrograms, and they allow somewhat speculative inferences about taxonomic levels and the time at which populations diverged *(27, 28)*. In addition, Hardy-Weinberg equilibria can be used to compare actual allozyme frequencies with those predicted for populations undergoing random mating *(29, 19)*. In the case of the African trypanosomes, this has provided strong evidence that *T. brucei* is a diploid, sexual organism. Although heterozygous patterns have been reported in *Leishmania* stocks *(30)*, there is as yet no such evidence for the leishmanias.

The complexities of some of the analyses outlined here may be daunting. Nevertheless, depending on study design, highly relevant questions can be clarified by simple visual comparison if sufficient enzyme characters are used.

Epidemiologic and Taxonomic Studies Using Isozymes

It is convenient to illustrate the types of studies that may be undertaken using isozymes, with particular reference to Brazil's Amazon Basin.

Arias *et al. (12)*, in studies of transmission cycles of *L. b. guyanensis*, required independent evidence that the sandfly vector and mammal reservoir had been correctly identified. Isolates from *Lutzomyia umbratilis* and the opossum, *Didelphis marsupialis*, that were presumed to be *L. b. guyanensis* were shown to be indistinguishable from human isolates with a minimum of eleven enzymes *(12)*. The demonstration that *D. marsupialis* was a reservoir host *(31)* was considered especially relevant to periurban outbreaks of cutaneous leishmaniasis. The same simple principle has been used to confirm that the sloth *(Choloepus didactylus)* and anteater *(Tamandua tetradactyla)* are major sylvatic reservoir hosts of *L. b. guyanensis (13, 32)*, and that the fox *Cerdocyon thous* carries an organism similar to the New World agent of visceral leishmaniasis *(33)*.

In a proportion of patients infected with *L. m. amazonensis*, the disease progresses to diffuse cutaneous leishmaniasis. The progression to this grave, incurable condition is thought to reflect some form of immunologic incompetence in the host rather than different strains of the parasite. Work relevant to this theory tested 30 Brazilian stocks of *L. m. amazonensis*. These were found to form a very homogeneous group, although they were collected from widely separated areas. Only two of 18 enzymes varied; and, in support of the above hypothesis, this variation was not associated with variation in the form of the disease *(34)*.

At least three distinct agents—*L. m. amazonensis, L. b. braziliensis*, and *L. b. guyanensis*—are considered to be responsible for cutaneous leishmaniasis in the Amazon Basin. *L. m. amazonensis* (Suprapylaria) is readily distinguishable from the two *L. braziliensis* subspecies (Peripylaria) on the basis of several biological characters *(2)*. However, distinction between *L. b. braziliensis* and *L. b. guyanensis* is more controversial, and is dependent upon epidemiologic factors and subtle growth characteristics in hamsters and *in vitro*. Distinction of *L. mexicana* from *L. braziliensis* by means of 10 out of 14 enzymes has confirmed the major differences between them. Slight mobility differences of four enzymes have also supported the separation of *L. b. braziliensis* from *L. b. guyanensis*, while indicating that they are closely related. Similarly, an extremely close relationship between *L. b. guyanensis* and *L. b. panamensis* has been indicated by a comparison in which 10 enzymes showed no differences between them *(23)*, although they could be distinguished by the enzymes phosphogluconate dehydrogenase (E.C.1.1.1.44, 6PGDH) and superoxide dismutase (E.C.1.15.1.1, SOD) *(23* and Lanham, personal communication).

Problems with the identity of a parasite may also involve more than one continent. New World visceral leishmaniasis is extremely similar epidemiologically to visceral leishmaniasis in the Mediterranean area of the Old World. Both have canine reservoir hosts, and it has been suggested that visceral leishmaniasis was introduced to South America with colonists through the importation of infected dogs *(35)*. Old and New World causative agents of visceral leishmaniasis, at present named *L. donovani infantum* and *L. d. chagasi*, respectively *(9)*, could not be distinguished by 10 enzymes *(36)*, a result confirming that they are very closely related. The fact that a parasite imported into South America should find an efficient vector in the sandfly *Lu. longipalpis* and attain a wide distribution is perhaps surprising, as is the benign infection of the native fox, *C. thous*, that would normally be taken to indicate a long-established zoonosis *(2, 33)*. This situation is analogous to that of the animal trypanosomiasis caused by *T. vivax* and *T. evansi*. Both have apparently spread from Africa but retain the same isozyme profiles *(25, 37)*. *T. evansi* also infects wild hosts in South America such as the capybara, *Hydrochoerus hydrochaeris (38)*.

Studies of animal leishmanias not known to cause disease in man have also been aided by the use of isozymes—for example, through sepa-

ration of *L. hertigi hertigi* and two groups of *L. h. deanei* parasites of the porcupine *(34)* and the discovery of a new *Leishmania* in the armadillo *Dasypus novemcinctus (39)*. Isozymes have not been used to fully investigate the relationships between other New World leishmanias, but preliminary data on *L. b. peruviana, L. m. mexicana, L. m. pifanoi,* and *L. m. aristedesi* are available and support the integrity of the taxa *L. m. mexicana* and *L. m. aristedesi*. Stocks designated *L. b. peruviana* have been found to have close affinities with *L. b. braziliensis*. Some stocks designated *L. m. pifanoi* have been found indistinguishable from *L. m. amazonensis,* and others have been found similar to *L. m. mexicana* (see Addendum). These results are based on limited comparisons with small numbers of stocks and require confirmation *(39)*.

In the Old World, although there is apparently not such an abundance of different leishmanias in small localities *(22)*, isozymes have played a similar role in helping to identify reservoir hosts in several regions *(40, 41, 42)*. Isozymes have also confirmed the separation of the two Old World cutaneous leishmaniasis complexes, *L. major* and *L. tropica (43, 44, 45)*. *L. aethiopica,* which is associated with diffuse cutaneous leishmaniasis in Ethiopia, has also proven identifiable by isozymes *(46)*, but it is not clear whether it deserves species *(26)* or subspecies *(9)* status. The close relationship between *L. donovani infantum* and *L. d. donovani* has also been supported by isozyme profiles *(36, 4, 5)*.

Excellent illustrations of the application of numerical taxonomy to the Old World *Leishmaniae,* using either similarity indices or allozyme frequencies and genetic distances, can be seen in the work of Lanotte *et al. (26)*. Both methods produced similar dendrograms that clearly delimited *L. major, L. tropica, L. aethiopica,* and *L. donovani*.

Other Biochemical Methods

There is a wide range of other biochemical procedures that are useful in taxonomic studies of leishmanias. With the possible exception of procedures using genetic probes and lectins *(47, 48)*, all depend on more sophisticated laboratory techniques, and none have been adapted to routine, large-scale identifications of the sort required by epidemiologic studies in endemic areas. Such potential or currently available methods include: determination of DNA buoyant densities (of both nuclear and kinetoplast DNA), DNA hybridization, comparison of DNA dissociation and reassociation curves, restriction endonuclease analysis of kinetoplast and nuclear DNA, comparisons of cell constituents and metabolic pathways, and two-dimensional SDS PAGE electrophoresis of proteins. These other biochemical taxonomic methods are reviewed in the proceedings of a special World Health Organization meeting to which interested readers are referred *(49)*. The particular promise of genetic probes, once a satisfactory substitute for the radiolabel is found, can be seen in the elegant study of Wirth and Pratt *(48)*.

Prospects

It is likely that methods of enzyme electrophoresis will be simplified still further, and a field kit based on cellulose acetate electrophoresis has been suggested *(17, 50)*. An international collection of standard stocks and clones may be developed to maintain the continuity of investigations and aid comparisons between different regions. More extensive use of isozyme profiles in epidemiologic studies will certainly occur, especially as culture of leishmanias from vectors and reservoir hosts becomes routine. There will also be corresponding changes in the classification of leishmanial parasites and a better appreciation of the relationships between Old and New World groups. In parallel with increased knowledge of the leishmanial genome, more detailed characterization of purified enzymes and their experimental use as genetic markers will permit further assessment of the presence of genetic recombination in *Leishmania*. Eventually, it is expected that the development of specific monoclonal antibodies *(14, 51)* or genetic probes *(48, 52)* will enhance and partially supersede the use of isozymes for identification.

ADDENDUM

Since the paper on which this article is based was presented (in November 1982), the classification of the genus *Leishmania* has been reviewed and significant progress has occurred in applying biochemical techniques to the identification of leishmanias. Interested readers are referred to the Proceedings of the International Symposium on Taxonomy and Phylogeny of *Leishmania* held in Montpellier, France, in July 1984 (*Annales de Parasitologie Humaine et Comparée*, in press). A review of the application of new technologies, including isozyme electrophoresis, to epidemiologic studies of trypanosomes has appeared (Gibson and Miles, *British Medical Bulletin* 41:115-121, 1985). In combination, the new method of pulsed field gradient gel electrophoresis and recombinant DNA techniques allow direct determination of the numbers, sizes, and homologies of *Leishmania* chromosomes and the mapping of genes (Van der Ploeg, et al., *European Journal for Molecular Biology* 3:3109-3115, 1985). The relationship between "molecular karyotype" and other methods of characterization, such as isozyme profiles, should soon become apparent.

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SUMMARY

Difficulties in classifying leishmania parasites according to their behavior in mammalian hosts and sandfly vectors have created a need to establish classification criteria that do not depend on these complex interactions. Isozyme electrophoresis provides a useful taxonomic tool for this purpose. Indeed, it has become the most widely applied biochemical procedure used to distinguish between leishmanias, and has made major contributions to understanding the epidemiologies of cutaneous and visceral leishmaniasis.

In essence, isozyme electrophoresis is accomplished by preparing crude, soluble extracts of the test organisms; applying these extracts to an electrophoretic plate; permitting the extract's component to move toward the cathode or anode in response to an electric charge; overlaying the plate with a specific substrate altered by the selected enzyme; and determining the movement of the enzyme on the plate by means of a staining procedure capable of revealing the location of the altered substrate. Differences in the resulting isozyme profiles suggest differences in the genes controlling production of the selected enzymes; and, if a sufficient number of enzymes are tested, the absence of such differences implies that the leishmania populations tested are very similar.

One of various complications involved in such testing is that mixed leishmania infections of mammals or vectors confuse the interpretation of isozyme data.

It is thus desirable that standard, representative *Leishmania* stocks be cloned for use in making broad isozyme comparisons.

At the same time, however, the availability of isozyme electrophoresis encourages description of isozyme profiles for leishmanias of doubtful origin and identity, sometimes with confusing results. Such descriptions, which could account for certain conflicting biological and biochemical observations in the literature, are ill-advised.

In general, it is recommended that initial investigations based on isozymes be performed in depth on a regional basis, and that they address specific, relevant epidemiologic issues. Continuity can be mantained with future studies by using representative *Leishmania* stocks throughout the comparisons. Such stocks will eventually provide a means for comparing isolates from diverse geographic regions. Ideally, at least for reference *Leishmania* strains, isozyme profiles should be used in conjunction with other methods of characterization such as molecular karyotyping or reactivity to monoclonal antibodies.

A number of studies provide examples of how isozymes have been used to date in *Leishmania* research. Specifically, isozyme analyses have provided corroborating evidence that the sandfly vector and mammalian reservoir of *Leishmania braziliensis guyanensis* have been correctly identified. They have been used to support the hypothesis that development of diffuse cutaneous leishmaniasis among people infected with *Leishmania mexicana amazonensis* reflects immunological incompetence in the host rather than infection with different parasite strains. They have helped to distinguish between *Leishmania braziliensis braziliensis* and *Leishmania braziliensis guyanensis*, and between *L. b. guyanensis* and *Leishmania braziliensis panamensis*. They have also served to confirm that the agents of Old World and New World leishmaniasis are closely related, have helped to identify reservoir hosts in several parts of the Old World, and have corroborated the distinctness of the two Old World cutaneous leishmaniasis complexes, *Leishmania major* and *Leishmania tropica*.

Overall, it is expected that future epidemiologic studies will make more extensive use of isozyme profiles, especially as culture of leishmanias from vectors and reservoir hosts becomes routine. This development should be accompanied by corresponding changes in the classification of leishmanias and an improved understanding of the relationships between Old and New World groups.

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