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SEROLOGIC INVESTIGATION OF HERPES SIMPLEX VIRUS: USEFULNESS OF THE DOUBLE IMMUNODIFFUSION (DID) TECHNIQUE IN EPIDEMIOLOGIC SURVEYS[1]

José Manuel Echevarría,[2] María Dolores Bermúdez de Castro,[3] Enrique Nájera,[4] and Rafael Nájera[5]

Testing of sera from 511 Spanish patients indicates that the double immunodiffusion technique is as sensitive as the neutralization test for detecting antibody to herpes simplex virus (HSV). The former method would also seem to have potential for distinguishing between antibodies to HSV types 1 and 2—a significant distinction because of the possible connection between HSV-2 and cancer of the human cervix.

Introduction

The double immunodiffusion (DID) technique has been used in recent years to study the antigenic composition of herpes simplex viruses (*1-4*). Nevertheless, little attention has been paid to the potential usefulness of this procedure for seroepidemiologic work or diagnosis of recent infections. Since the DID technique is simple and yields quick results compared to the neutralization (N) and complement fixation (CF) tests, it would be of interest to know how similar its results are to those of these other techniques—in order to gauge its potential usefulness for seroepidemiology and diagnosis. The work reported here had two purposes: to examine the distribution of neutralizing, precipitating, and complement-fixing antibodies to the herpes simplex viruses in the Spanish population, and to compare the results obtained by the DID technique with those obtained by the N and CF methods.

Materials and Methods

Viruses and Antigens Employed

The antigen used in all the tests was herpes simplex virus type 1 (HSV-1), strain HFEM, obtained from an Australian subject (Hilda F.) in 1937. P. Wildy, who cloned this strain three times consecutively in chick embryo chorioallantoic membranes in 1953, graciously supplied us with this material.

To reproduce the virus—so as to obtain antigen lots for DID, N, and CF testing—the strain was inoculated onto confluent monolayers of BHK-21* cells grown in Winchester flasks. After allowing 2 hours for adsorption (at 37°C), enough medium 199 (maintenance medium for tissue culture) was added to bring the total volume to 200 ml. Later, when a generalized cytopathic effect was observed (normally in 24

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[2] Assistant, Respiratory Virus Service; National Center for Sanitary Microbiology, Virology and Immunology; Madrid, Spain.

[3] Chief, Cell Cultures Section; National Center for Sanitary Microbiology, Virology, and Immunology.

[4] Chief, Epidemiology Service; National Center for Sanitary Microbiology, Virology, and Immunology.

[5] Chief, Respiratory Virus Service; National Center for Sanitary Microbiology, Virology, and Immunology.

*Baby hamster kidney.

to 48 hours) the cells were detached from the glass of the flask with a rubber scraper, centrifuged at 700 revolutions per minute (rpm) for 20 minutes, resuspended in 2 ml of medium 199, and sonicated for 20 seconds at 60 watts in a B-12 Sonifier (Branson Sonic Power Co., Danbury, Connecticut). The extracts of infected cells thus obtained were centrifuged again at 700 rpm for 30 minutes to eliminate membrane fragments, and the resulting clear supernatent fluid was used as antigen in the DID and N tests. Antigen for the CF test was prepared from these cell extracts by treating them with 5 per cent chloroform, incubating them for 20 hours at 4°C, and centrifuging them at 700 rpm for 15 minutes. Between preparation and use, all the antigens were stored undiluted at -70°C.

Cell Cultures

VERO cells, used as indicators of neutralization in the N tests, were grown on Eagle's Minimum Essential Medium (MEM) in Earl's saline containing 5 per cent calf serum (Bio-Cult Laboratories, Scotland), 20,000 units/ml of penicillin, 10 mg/ml of streptomycin, and 0.1 mg/ml of NaOH. Until used in the N tests, these cultures were maintained by successive passages—in which the cells were dispersed by treatment with a 9.25 per cent trypsin solution (Difco, 1:250) and with 0.02 per cent EDTA in PBS,* until complete dispersal of the monolayer was achieved.

The BHK-21 cell monolayers used for production of antigen were grown on the same medium as described above, except that bovine fetal serum (Flow Laboratories, Irvine, Scotland) was substituted for calf serum.

*Edetate-ethylenediaminotetra-acetate; phosphate buffered saline.

Neutralization (N) Tests

These tests employed the Takatsy microtechnique (1954) as modified by Sever in 1962 (*5*), following the guidance provided by Kalter in 1973 (*6*). The tests were performed with presterilized cell culture microtiter plates (see photo) of the "Cook Microtiter" type (C.A. Greiner, West Germany). The test sera were titered without prior inactivation.

Complement Fixation (CF) Tests

These tests utilized the 1974 Grist technique (*7*), a modification of the procedure described by Bradstreet and Taylor in 1962 (*8*), and employed U-plates (model IS-MAC-96) manufactured by the Linbro Scientific Co. in Hamden, Connecticut.

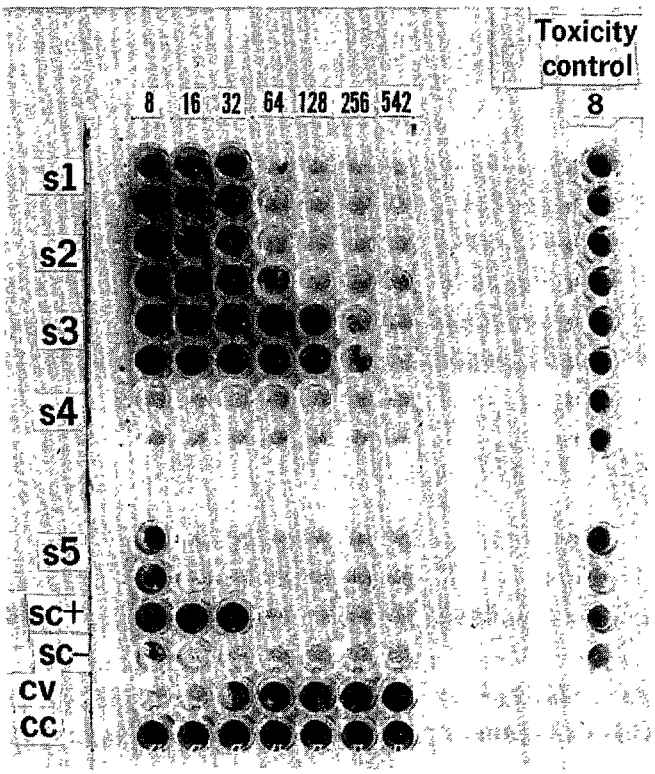
Double Immunodiffusion (DID) Tests

Glass 9 x 8 cm plates and plastic 7 x 2 cm slides (Div. Travenol Laboratories, California) were used in these tests. The gel employed was made up with a 1 per cent suspension of Ionagar (Difco) in distilled water containing 0.9 per cent NaCl and 0.1 per cent sodium azide. The thickness of the gel on the plates was 2 mm.

The sera were put in wells 4 mm in diameter that were regularly arranged, in groups of four or six, around a central well the same size—where the antigen was placed. The distance between the edge of the central well and the nearest edge of each peripheral well was 2 mm.

The wells were inoculated with .03 ml of serum or antigen, and the antigen was allowed to diffuse a little, after which .025 ml of a 2 per cent sodium lauryl sarcosinate solution in distilled water was added to each antigen well. The plates were then left in a humid chamber at room temperature for 48 hours, after which they were washed by immersion in 0.85 per cent NaCl for 24 to 48 hours. They were then

Neutralization testing of sera with HSV-1 antigen on microtiter plates.



stained by immersion for 5 minutes in a solution of 1 per cent amido Schwartz stain (prepared with 6 per cent glacial acetic acid and 1.36 per cent sodium acetate) in distilled water. Excess stain was removed by washing the plates in a solution of 5 per cent glacial acetic acid and distilled water for 24 hours. As shown in the photograph, control samples of antigen (Agc), positive sera (sc+), and negative sera (sc-) were included in each test. The control antigen was prepared with BHK-21 cells from uninfected cultures, using the same method employed to prepare the extracts of infected cells that were used as antigen.

Test Sera

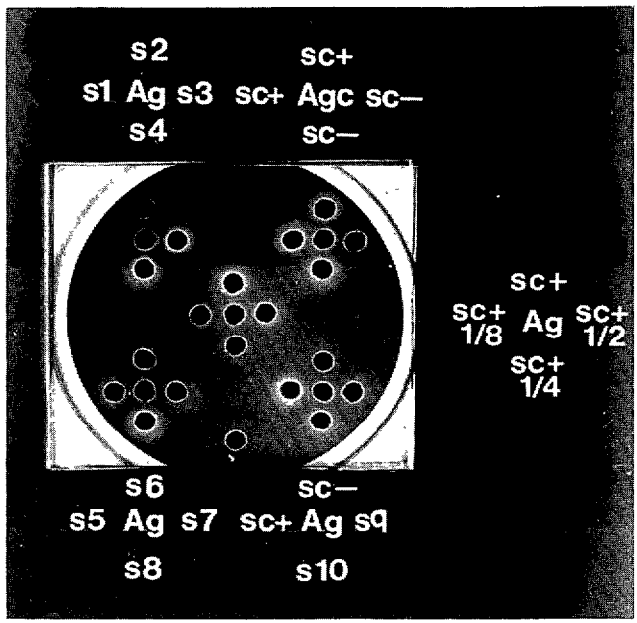
A total of 511 sera from healthy individuals or patients with nonherpetic conditions were used in the survey. The sera, chosen from the clinical samples received by our respiratory virus service during 1974-1976, came from various provinces of Spain. They were stored at -20°C from the time of receipt until use and were divided into 12 groups according to their respective subjects' age.

Results

The results of the N and DID tests are shown in Table 1 and Figure 1. As the figure indicates, after showing a rapid decline during the first 7 to 8 months of life, the percentage of positive sera[6] rose sharply

[6]Sera yielding a clean precipitation band in the DID test, a neutralization titer $\geq$1:8 in the N test, or a titer $\geq$1:8 in the CF test were considered positive by each of these respective methods.

Double immunodiffusion testing of sera with HSV-1 antigen on glass 9 × 8 cm plates.



sc+ = positive control serum
sc- = negative control serum
Ag = antigen
Agc = control antigen

Table 1. Percentages of sera yielding positive N and DID test results, by age group.

Age group	No. of sera tested	Positive sera (N test)		Positive sera (DID test)		95 per cent confidence limits for N test results	95 per cent confidence limits for DID test results
		No.	%	No.	%		
0-1 month	49	42	85.7	40	81.6	72.8-94.1	70.0-91.2
1-6 months	31	22	71.0	15	48.4	52.0-85.8	31.1-67.0
6-12 months	26	4	15.4	3	11.5	4.3-34.9	2.4-30.1
1-2 years	20	7	35.0	5	25.0	15.3-59.2	8.6-49.1
2-5 years	46	27	58.7	26	56.5	43.2-73.0	41.1-71.1
5-10 years	42	21	50.0	19	45.2	34.2-65.8	29.8-65.3
10-15 years	49	37	75.5	37	75.5	61.1-86.6	61.1-86.6
15-20 years	50	44	88.0	42	84.0	75.7-95.5	70.9-92.8
20-25 years	48	34	70.8	33	68.8	55.9-83.0	53.7-81.3
25-30 years	50	38	76.0	38	76.0	61.8-86.9	61.8-86.9
30-40 years	50	45	90.0	45	90.0	78.2-96.7	78.2-96.7
40-80 years	50	48	96.0	48	96.0	86.3-99.5	86.3-99.5
0-80 years	511	369	72.2	351	68.7		

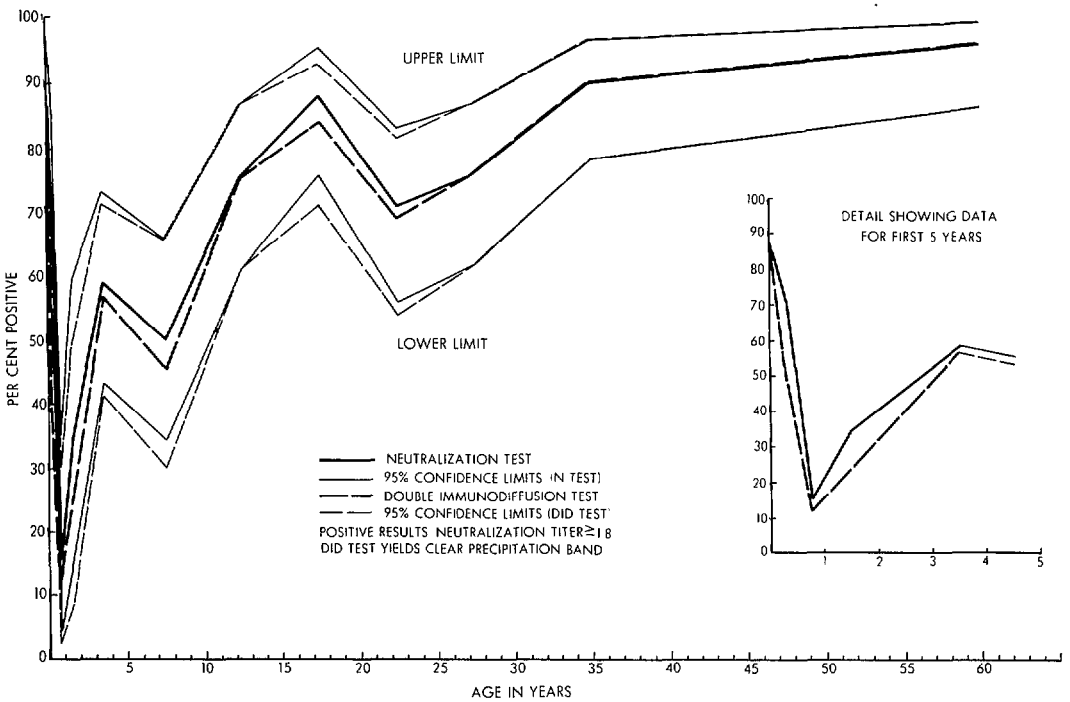


Figure 1. Percentages of sera from different age groups yielding positive N and DID test results. The thin lines show 95 per cent confidence limits for these data.

until the subjects reached 2-5 years of age. Then, from 5-10 years onward it continued rising, but more slowly, until the subjects were 15-20 years of age. Finally, beyond the 20-25 year group there was a very slow rise that ended, in the oldest age group, with 96 per cent of the subjects yielding seropositive results.

Of the sera giving positive DID results, 200 (57 per cent) showed a single precipitation band and 151 (43 per cent) showed two bands. Of the sera yielding a double band, only 23 (15 per cent) were obtained from subjects between 6 months and 15 years of age. These results are shown in Table 2 and Figure 2.

The CF test results are shown in Table 3 and Figure 3. The percentage of positive sera was found to decline rapidly at first, falling to 8 per cent in the 6-12 month age group. After that it rose rapidly (exceeding 40 per cent in the 2-5 year age group), remained at about the same level in the 5-10 and 10-15 year age groups, dropped slowly to 24 per cent in the 20-25 year group, and then rose slowly again, reaching a plateau around 51 per cent among subjects above 30 years of age. This latter percentage is roughly comparable to the 62 per cent positivity found in the sera from subjects less than 1 month old.

Discussion

The curves obtained with the N and DID techniques are clearly similar and, indeed, virtually identical—a fact confirmed by estimating the 95 per cent confidence limits of these results. None of the differences in the results of the two tests—with respect to each of the age groups involved—were found to be statistically significant. It is worth noting, however, that the greatest difference between the tests oc-

Table 2. Percentages of sera yielding two precipitation bands upon DID testing, as compared to sera yielding positive DID test results, by age group.

Age group	No. of sera tested	Sera yielding positive DID test results		95 per cent confidence limits for DID test results	Sera showing 2 precipitation bands		95 per cent confidence limits for sera showing 2 bands
		No.	%		No.	%	
0-1 month	49	40	81.6	70.0-91.2	16	32.6	19.9-47.5
1-6 months	31	15	48.4	30.1-67.0	4	12.9	3.6-29.8
6-12 months	26	3	11.5	2.4-30.1	1	3.8	0.1-19.6
1-2 years	20	5	25.0	8.6-49.1	1	5.0	0.1-24.9
2-5 years	46	26	56.5	41.1-71.1	5	10.9	2.4-20.8
5-10 years	42	19	45.2	29.8-65.3	9	21.4	10.3-36.8
10-15 years	49	37	75.5	61.1-86.6	7	14.3	5.9-27.2
15-20 years	50	42	84.0	70.9-92.8	21	42.0	28.2-56.8
20-25 years	48	33	68.8	53.7-81.3	18	37.5	24.0-52.6
25-30 years	50	38	76.0	61.8-86.9	20	40.0	26.4-54.8
30-40 years	50	45	90.0	78.2-96.7	29	58.0	43.2-71.8
40-80 years	50	48	96.0	86.3-99.5	20	40.0	26.4-54.8
0-80 years	511	351	68.7		151	29.5	

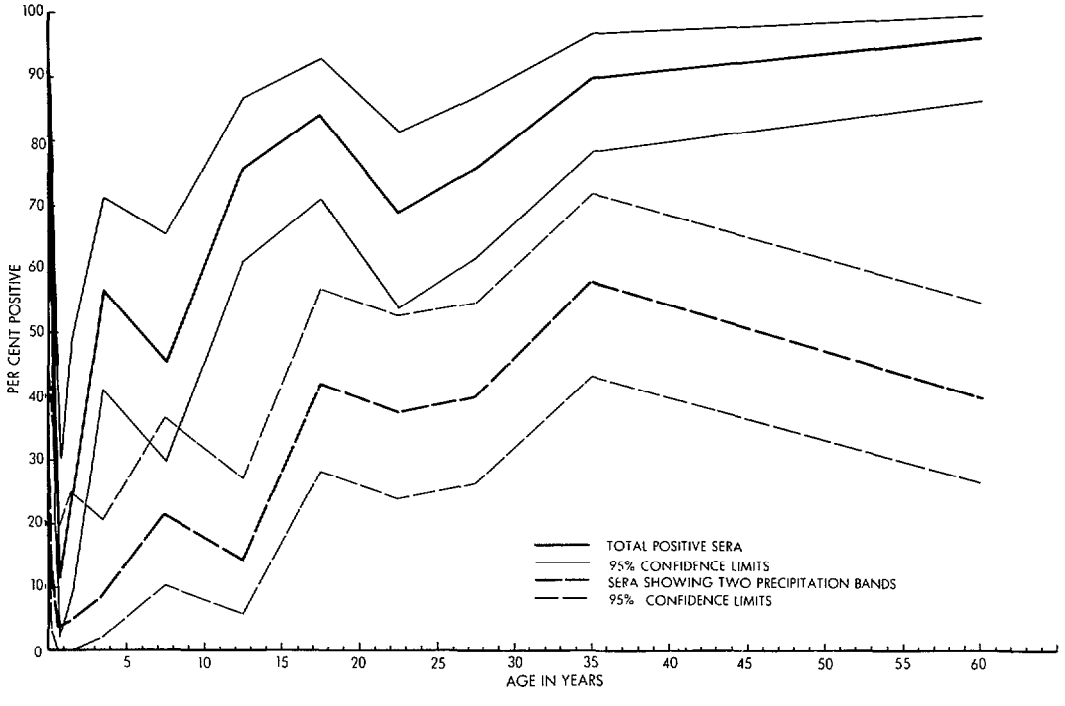


Figure 2. Percentages of sera from different age groups yielding positive results and two precipitation bands on DID testing. The thin lines show 95 per cent confidence limits for these data.

Table 3. Percentages of sera yielding positive CF test results (titers ≥ 1:8), by age group.

Age group	No. of sera tested	Positive sera (titers ≥ 1:8)		95 per cent confidence limits of CF test results
		No.	%	
0-1 month	48	30	62.5	47.3-76.0
1-6 months	31	6	19.3	7.4-37.5
6-12 months	24	2	8.3	1.0-27.0
1-2 years	19	3	15.8	3.4-39.5
2-5 years	46	19	41.3	27.0-56.7
5-10 years	42	18	42.9	27.7-59.0
10-15 years	48	20	41.7	27.6-56.8
15-20 years	48	18	37.5	24.0-52.6
20-25 years	46	11	24.0	12.6-38.8
25-30 years	47	15	31.9	19.0-47.1
30-40 years	43	22	51.2	35.5-66.7
40-80 years	45	23	51.1	35.8-66.3
0-80 years	487*	187	38.4	

*Twenty-four sera (out of 511 samples) were not tested by CF (4.6 per cent of the total) because of lack of sera in these cases.

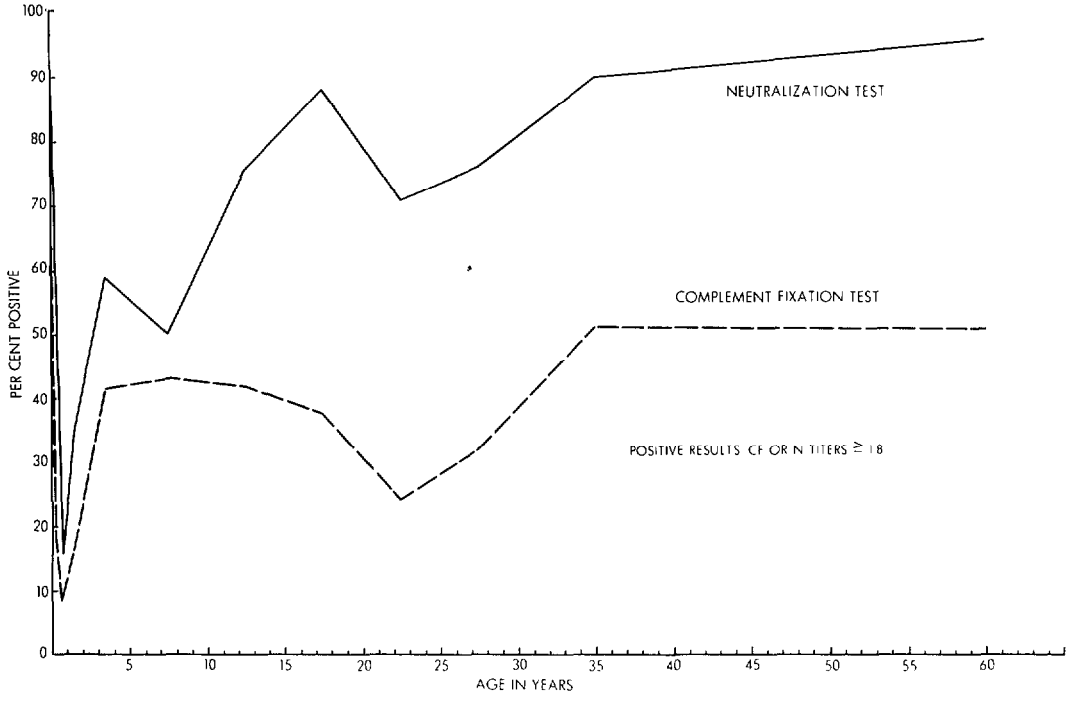


Figure 3. Percentages of sera from different age groups yielding positive N and CF test results.

curred in sera from subjects 1-6 months of age—at a time when antibodies acquired from the mother were disappearing and when the highest percentage of low neutralizing titers (less than or equal to 1:8) were detected.

In view of the fact that the DID test was capable of detecting the sera yielding neutralization titers $\geq$ 1:8, these results clearly indicate very similar levels of sensitivity. In addition, the DID test employs far simpler procedures than the N test, and unlike the latter requires no sterile materials or maintenance of cell cultures. Therefore, it is felt that the DID technique could prove useful in seroepidemiologic studies of herpes simplex virus and also in laboratory diagnosis of recent herpes simplex infections.

As Figure 2 indicates, there is some evident difference between the overall percentages of sera yielding positive DID results and the percentages yielding two precipitation bands. The chart also shows that in terms of subject age groups there may have been some time-lag involved in development of seropositivity with two precipitation bands as compared with one. Because of the small numbers of sera tested in each age group, the confidence limits of the two curves were too broad to confirm existence of this time-lag. If the apparent lag is real, however, it could be accounted for in either of the following ways:

- The second band could arise from a component of the same virus that is less antigenic or more difficult to detect than that responsible for the first band.
- The second band could be specific for a particular type of herpes simplex virus. Since the curve in Figure 3 showing the percentage of double-band sera in each age group appears to lag somewhat behind that showing the overall percentage of positive sera in each group, it appears plausible that the second band could be specific for type 2 herpesvirus (HSV-2), i.e., that it could have arisen from a HSV-2 component cross-reacting with our HSV-1 antigen because this antigen is not type-specific.

One particular precipitation band was observed in all the sera that yielded positive DID results. In 1974 Skinner et al. (*1*) described one precipitation band, called "Band II," as being common to all human sera reacting positively when tested by DID against preparations of either type of herpes simplex virus. To find out whether our common band corresponded to this one, 50 of the positive sera were tested in parallel with "Band II" antiserum graciously supplied by Dr. Skinner. In all cases the bands produced by the antiserum and by the test sera were found to be the same. Hence it could be concluded that this common band represents "anti-Band II" antibodies.

The CF test results clearly reinforce those of the N and DID tests. All the results suggest that herpes simplex primoinfection occurs most commonly between the first and fifth years of life, since all three tests showed a rapid rise during these years in levels of seropositivity. Among older age groups the level of CF positivity declined slightly, but the level rose again among subjects over 25 years of age and then remained stable at about 50 per cent for subjects over age 30. This behavior of the CF curve is highly consistent with the fact that the N and DID curves never clearly leveled off but continued to rise, even though the rise was relatively slight after 5-10 years of age.

Either of two hypotheses could account for the second rise of the CF curve:

1) It may be that herpes simplex primoinfection is most common in the first five years of life, after which it falls off, and that its effects are reinforced among older age groups by recurrences, which in some cases prompt the reappearance of complement-fixing antibodies.

2) Backward (and downward) projection of the second rise in the CF curve intersects the horizontal axis at a place corresponding to 10-12 years of age. This could indicate that around that age HSV-2 infection

begins to be detected, so that in effect the curve obtained is the sum of two curves—each indicating antibodies to one of the two types of virus. That is, during the first 10 or 12 years of life we would see a curve resulting exclusively from circulation of HSV-1, and after that we would see a curve resulting from circulation of both HSV-1 and HSV-2. This hypothesis appears consistent with the available data on surveys carried out in healthy populations using the kinetic neutralization test (*9, 10*). The results of these surveys indicate that specific HSV-2 neutralizing antibodies begin to appear around 13 years of age and reach positivity levels of 20 per cent in groups of subjects above 20-25 years of age.

Differentiation of HSV-1 and HSV-2 antibodies in human sera is a subject that has aroused great worldwide interest because of the possible connection between HSV-2 and cancer of the human cervix. Considerable emphasis is therefore being placed on the need for a quick and simple technique capable of distinguishing the two types of HSV.

The results presented here clearly show that the DID technique is fully applicable to detection of antibodies against HSV in human sera at a level of sensitivity comparable to that attained by the neutralization test. Moreover, at this point it appears that a DID test improved in terms of sensitivity could help to identify some of the polypeptides described as carriers of total or partial type-specificity—such as VP19e (*11*) or VP7 and VP8. This would make it possible to use the DID technique for detection of type-specific antibodies, thereby taking advantage of that technique's simplicity and speed.

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SUMMARY

The work reported here was designed to test the potential usefulness of the double immunodiffusion technique for seroepidemiologic and diagnostic work on herpes simplex virus (HSV).

A total of 511 sera from subjects attending Spain's Respiratory Virus Service in 1974-1976 were tested for HSV antibodies by double immunodiffusion (DID), neutralization (N), and complement fixation (CF). The DID test results closely paralleled those of the N test, both of these detecting considerably more positive sera than the less sensitive CF test.

In addition, some of the positive sera tested by DID yielded two precipitation bands. While the second band could have arisen merely from a viral component that was less antigenic or harder to detect, it could also have been produced by antibody to a particular type of HSV. Specifically, it seems possible that the second band could have been caused by antibody to a HSV type 2 component cross-reacting with the HSV type 1 test antigen employed.

Overall, the results clearly show that the DID technique is as sensitive as the N test with respect to detection of HSV antibodies. They also suggest that an improved DID test might be developed that could help distinguish between antibodies to HSV-1 and HSV-2.

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BIOLOGICAL CONTROL OF DISEASE VECTORS*

The World Health Organization is encouraging and coordinating international research on the biological control of vectors as part of a special program of research and training in tropical diseases. The program is the outcome of a collective effort by many countries and international agencies to make better use of existing control methods, to train personnel, and to develop research on these diseases.

Specialized scientific working groups deal with the development of new control methods for each group of tropical diseases, in particular leprosy, malaria, filariasis, schistosomiasis, trypanosomiasis, and leishmaniasis, or are responsible for activities, such as the biological control of vectors that cover all or most of these diseases.

The objectives of the Working Group on the Biological Control of Vectors are to identify, evaluate, and develop biological control agents for the safe and effective control of invertebrate vectors and intermediate hosts of human diseases, with special emphasis on bacteria, fungi, protozoa, and nematodes.

The Steering Committee of the Working Group has drawn up a plan of action for 1980 and 1981 in the light of the latest developments in biological control and the expected progress in research in this field.

*Source: WHO, In Point of Fact, No. 10/1980.