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Enzyme Immunoassay Versus Plaque Neutralization and Other Methods for Determination of Immune Status to Measles and Varicella-Zoster Viruses and Versus Complement Fixation for Serodiagnosis of Infections with Those Viruses

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Results by an enzyme immunoassay method (EIA) performed at one serum dilution and results by indirect immunofluorescence (IFA) and hemagglutination inhibition (HI) tests performed at step dilutions were correlated with results by a neutralization test (50% plaque neutralization [PN]) performed at step dilutions on single serum samples for serologic evaluation of immunity status to measles virus. PN results were taken as true indicators of immunity, and the other tests were evaluated on that basis. The predictive value of a positive result being positive also by PN was 95.3% for HI and 93.3% for EIA and IFA. The predictive value of a negative result being negative also by PN was 81.1% for HI, 100% for EIA, and 75.0% for IFA. A similar study on immunity status to varicella-zoster virus by EIA and by an anticomplement immunofluorescence test versus PN showed a 100% predictive value of a positive or negative result by EIA. By the anticomplement immunofluorescence test, the predictive value of a positive result was 97.7%, and that of a negative result was 88.5%. Studies on the comparative ability of EIA versus complement fixation (CF) to detect significant changes in antibody concentration between acute-phase and convalescent-phase serum samples indicative of a current infection were also done. Both tests were satisfactory for the serodiagnosis of measles or varicella-zoster virus infections. However, EIA was preferable to CF because it was less technically difficult, less labor intensive, and could be performed on sera that were anticomplementary in CF reactions.

The most meaningful test for the serologic evaluation of immunity status to viral infections is the neutralization test. However, it is not a feasible test to use on a routine basis because of the expense of tissue culture, the amount of labor involved in performing the test, and the length of time before results are obtained. The next best serologic approach is to compare results of neutralization tests with those of other tests and to select for use the test showing the highest correlation with the neutralization test. In the present report, data are presented on the correlation of plaque-neutralizing (PN) antibody to measles and varicella-zoster viruses with antibody detected by enzyme immunoassay (EIA), immunofluorescence methods, and complement fixation (CF) and also by hemagglutination inhibition (HI) for antibody to measles virus. Comparisons were also made between EIA and CF for the diagnosis of current infection with these viruses.

MATERIALS AND METHODS

CF antigens. Viral antigens were extracted from infected cell cultures (HeLa cells infected with measles virus, Edmonston strain, or human fetal diploid lung cultures infected with varicella virus, Batson strain) when the cytopathic effect was 4+ by one (varicella-zoster virus) or two (measles virus) 30-s periods of sonic treatment (20 kHz; Biosonic II, Bronwill Scientific Inc., Rochester, N.Y.). Supernatant fluids after centrifugation of the cell lysates (800 × *g* for 15 min at 5°C) were used as CF antigens. Control antigens were prepared in the same manner for noninfected cell cultures.

EIA antigens. (i) Measles virus. Cells from a human fetal diploid line (HFDL-645) infected with measles virus (Edmonston strain) and showing 4+ cytopathic effect were resuspended in Hanks balanced salt solution to 1/20 of their original culture fluid volume. The cell suspension was kept frozen at −70°C until further processed. The antigen was extracted by treatment of the cell suspension with an equal volume of 0.2% deoxycholate (12) in 0.005 M phosphate-buffered saline (pH 7.2; PBS) with intermittent mixing at 5°C. After clarification of the cell suspension at 2,200 × *g* for 15 min at 5°C, the supernatant fluid was centrifuged at 28,000 × *g* for 90 min at 5°C. The supernatant fluid from this centrifugation was used as the antigen. A similar preparation was made from noninfected cells for use as a specificity control.

(ii) Varicella-zoster virus. HFDL-645 cells infected with varicella-zoster virus (Batson strain) and showing 3 to 4+ cytopathic effect were suspended in 0.1 M glycine buffer (pH 9.5) at 1/10 of the volume of the original culture fluid. The cell suspension was subjected to three 10-s periods of sonic vibration at 50% probe intensity (Biosonic II) and then centrifuged at 569 × *g* for 15 min at 5°C. The supernatant fluid was used as the antigen after treatment with 4′-aminomethyl-4,5′,8-trimethyl psoralen (10 μg/ml) and long-wave UV irradiation at 1.9 mW/cm^2 for 8 min at 5°C to inactivate any residual virus (7). A similar preparation was made from noninfected HFDL-645 cells for use as control antigen.

EIA. Trays of cuvettes with a 1-cm light path (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) were coated with 0.25 ml of the viral antigens diluted 1:75 (measles virus) or 1:150 (varicella-zoster virus) in 0.005 M PBS (pH 7.2), dilutions previously determined to be optimal by block titration with standardized reference sera. Alternate cu-

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vettes in the tray were coated with the appropriate control antigen at the same protein concentration as that of the corresponding viral antigen as determined by the Lowry method. The cuvettes were dried by a fan at 37°C overnight. They were then packaged in airtight polyethylene bags (Belart Plastic, Pequannock, N.J.) containing silica gel desiccant (Dri Pack Bags; Davison Chemical, Baltimore, Md.) and a humidity indicator card and stored at 5°C until used. (Coated cuvettes stored for up to 6 months remained satisfactory for use, providing there was no change in the humidity indicator card.) All subsequent washes and additions of reagents, except for the serum samples, were automatically delivered and aspirated with a programmable PR50 processor/reader (Gilford). The coated curvettes were washed with 8 cycles of PBS containing a final concentration of 0.05% Tween 20 (0.7 ml per cycle) before use, after reaction with the serum samples, and after reaction with enzyme-labeled conjugate. Duplicate serum samples at a 1:100 dilution in PBS-Tween 20 containing 0.5% bovine serum albumin were incubated in the coated cuvettes (0.25 ml per cuvette) for 90 min at room temperature. After washing of the cuvettes, the $F(ab')_2$ fragment of goat anti-human immunoglobulin G (IgG) labeled with alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was added at a dilution of 1:1,500 in PBS-Tween 20-bovine serum albumin, an optimal dilution determined by block titration with reference sera. The $F(ab')_2$ fragment was used because the reaction of some serum samples with control antigen was less with the labeled $F(ab')_2$ fragment than with the labeled whole IgG molecule. After an incubation period of 90 min at room temperature, the cuvettes were washed, and the substrate (*para*-nitrophenylphosphate; 1 mg/ml in diethanolamine buffer [pH 9.8] containing 0.0005 M $MgCl_2$ and 0.02% sodium azide) was added. All reactions were automatically read, and results were calculated by a Hewlett Packard HP85B computer interfaced with the PR50 processor when the reaction of a reference serum reached a predetermined optical density (OD). In each run, in addition to this reference serum, samples of high-positive, low-positive, and negative sera were included. The OD of the reaction with the control antigen was subtracted from that with the viral antigen, and the average of the corrected ODs was calculated. The OD range and the mean of reactions with control antigen for varicella-zoster virus were 0.11 to 0.35 and 0.201, respectively, and for measles virus they were 0.11 to 0.39 and 0.196, respectively.

Determination of cutoff values for EIA. A total of 30 sera negative for measles virus antibody by indirect immunofluorescence (IFA) and 38 sera negative for varicella-zoster virus antibody by anticomplement immunofluorescence (ACIF) were checked by EIA. The means of the ODs plus 3 standard deviations for the reactions with measles and varicella-zoster viruses were 0.0975 and 0.0662, respectively. An OD of 0.1 was taken as a cutoff for a positive reaction with both viruses. Results were reported as an index: index = OD with viral antigen − OD with control antigen/cutoff OD (0.1).

To determine a cutoff indicating a significant difference in antibody concentration between paired serum samples, 53 serum pairs with standing antibody titers by CF to measles virus and 30 serum pairs with standing CF titers to varicella-zoster virus were tested in duplicate by EIA. The relationship between titers of paired serum samples is called a standing titer when the titers of acute- and convalescent-phase serum samples are the same or differ by no more than one doubling dilution. The index of each sample was calculated, and the ratio of the index of the second sample to the index of the first sample was determined. The mean plus 3 standard deviations of the ratios for antibody to measles virus was 1.307, and to varicella-zoster virus it was 1.274. A cutoff ratio of 1.50 was therefore taken as the ratio for a significant difference in antibody concentration between paired samples for both viruses and indicated a current infection.

HI, CF, and ACIF. HI and CF tests were done by standard methods (6, 8) with starting serum dilutions of 1:4 for HI and 1:8 for CF. The IFA with fluorescein-labeled rabbit anti-human IgG (not heavy chain specific; Beckman Instruments, Inc., Fullerton, Calif.) was employed for checking antibody to measles virus at a starting serum dilution of 1:8. To avoid the problem of Fc receptors, the ACIF test was performed as described before (13) by using fluorescein-labeled goat anti-guinea pig complement (anti-C3 component; Cappel Laboratories, Cochranville, Pa.) to test for antibody to varicella-zoster virus. Sera were checked by ACIF at a starting dilution of 1:4. The cell smears used for detection of antibody by immunofluorescence were prepared by mixing one part of cells infected with the virus under study and two parts of noninfected cells which served as nonspecific controls. The tests were read by epifluorescence microscopy with Zeiss equipment.

PN test. (i) Measles virus. Vero cells (an African green monkey kidney cell line) growing as monolayers in 24-well tissue culture plates (Costar, Cambridge, Mass.) were inoculated with measles virus-serum mixtures containing increasing dilutions of serum and a constant amount of virus (35 to 50 PFU [final concentration] per inoculum). Before inoculation, the virus-serum mixtures were incubated for 1 h at 5°C. After an adsorption period of 1 h at 36°C in an atmosphere of 5% CO_2 and 95% air, the monolayers were overlaid with soft agarose (equal volumes of 1% agarose and 2× Eagle minimum essential medium in Earle balanced solution and containing 2% fetal bovine serum), and incubation was continued. After 7 days, the monolayers were overlaid with a second layer of soft agarose in Eagle medium-Earle solution containing 0.01% neutral red. After an additional 24 h of incubation, plaques were counted. The endpoint was taken as 50% PN. All samples were tested in duplicate. Virus-inoculated cells and normal cell controls were included in each run.

(ii) Varicella-zoster virus. Neutralizing antibody was measured by a PN test by using approximately 10 hemolytic units of guinea pig complement in the serum-virus mixture as previously described (14). The endpoint was taken as 50% PN.

Serum samples. All serum samples were submitted to our laboratory either for diagnosis of a possible current infection with varicella-zoster or measles virus or for determination of immunity status to these viruses. The sera for comparative studies on tests for immunity status to varicella-zoster virus were from a single group of 66 teenagers. The 113 sera for comparison studies on tests for immunity status to measles virus were selected from a larger bank of 376 sera on the basis of EIA results. In the selection, emphasis was placed on samples with a low EIA index (1 to <5). Such sera were the least plentiful and represented 16.2% of the serum samples in the larger bank. Indexes of the remaining samples, when antibody was present, were in the middle (index, 5 to <10; 20.7%) to high (index, ≥10; 43.6%) range of values. Negative samples accounted for 19.4% of the total.

Sucrose gradient centrifugation. IgG in certain serum samples was separated from IgM by centrifugation of 0.4 ml of a

1:2 dilution of serum on a 10 to 40% linear gradient in a no. 41 Beckman rotor for 20 h at 5°C. Fractions removed from the bottom of the gradient were checked by Ouchterlony analysis with anti-IgM and anti-IgG (heavy chain specific; Behring Diagnostics, Sommerville, N.J.).

RESULTS

Correlation of results by various tests with results by PN for determination of immunity status. (i) Immunity to measles virus. The cutoffs for a positive result for presence of antibody by the various methods were: an antibody titer of 1:4 by HI and PN; an antibody titer of 1:8 by IFA; an antibody index of 1.0 by EIA at a 1:100 serum dilution. Results by PN were taken as the true measure of the immunity status of an individual, and all other tests were evaluated on that premise. The range and frequencies of EIA serum indexes selected for study and their correlation with the presence or absence of antibody by PN are shown in Fig. 1.

EIA results gave the highest predictive value (100%) that a negative reaction for antibody to measles virus was a true-negative result (all samples negative by EIA were also negative by PN) (Table 1). The predictive values of negative results by HI and by IFA were 81.1 and 75.0%, respectively. The predictive value that a positive result was a true-positive result was 95.3% by HI and 93.3% by both EIA and IFA.

Serum samples that were positive for antibody by EIA or IFA (six samples each) or by HI (four samples), but had no neutralizing antibody by PN, were identified to see whether the same or different sera were involved. All of these sera, except one positive by EIA (index, 1.70) and one positive by IFA (serum titer, 1:32), were also positive by one or both of the other tests (Table 2).

(ii) Immunity to varicella-zoster virus. The cutoff for a positive result for antibody to varicella-zoster virus was a 1:4 titer by ACIF and an index of 1.0 by EIA at a 1:100 serum dilution. Figure 2 shows the range and frequencies of the indexes of the tested sera and their correlation with the presence or absence of neutralizing antibody. Based on PN results as true indicators of the presence or absence of immunity, the predictive value of a positive or negative result by EIA was 100% (Table 3). By ACIF, the predictive values of positive and negative results were 97.7 and 88.5%, respectively.

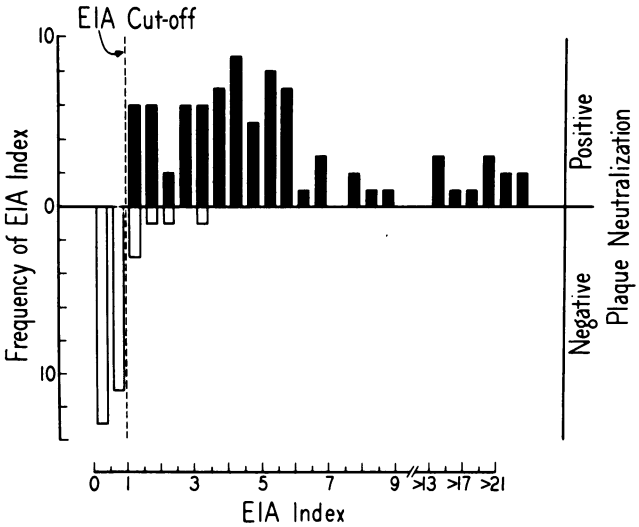

FIG. 1. Frequency of EIA indexes of the serum samples selected for study of immune status to measles virus and the correlation between EIA and PN results. Vertical dashed line (EIA cutoff) separates positive EIA results (index, ≥1.0) from negative EIA results (index, <1.0). Solid bars represent positive results by PN (titer, ≥1:4); open bars represent negative results by PN (titer, <1:4).

TABLE 1. Comparison of EIA, HI, and IFA with PN for determination of immunity status with measles virus

Test and result	No. of PN results		Predictive value of results (%)[a]	
	Positive	Negative	Negative[b]	Positive[c]
EIA				
Positive	83	6		93.3
Negative	0	24	100	
IFA				
Positive	73	6		93.3
Negative	10	24	75.0	
HI				
Positive	75	4		95.3
Negative	7	26	81.1	
Nonspecific	1			

[a] Predictive values of true-positive or -negative results were based on PN results, which were taken as true indicators of immunity.
[b] Predictive value of a negative result = true-negative results/true-negative results + false negative results.
[c] Predictive value of a positive result = true-positive results /true-positive results + false-positive results.

Comparative studies between EIA and CF for serodiagnosis of current infection. (i) Infection with measles virus. Complement fixation, although generally not useful for the detection of antibody from past infections with measles or varicella-zoster viruses, is a valuable test for the serodiagnosis of current infections. It was of interest, therefore, to determine whether EIA was as useful as CF in this regard. A total of 255 serum pairs submitted for the serodiagnosis of measles were tested by both tests. A significant increase in antibody concentration between the acute- and convalescent-phase samples, indicative of a current infection, was a fourfold or greater increase in antibody titer by CF and a ratio of the indexes of the convalescent- to acute-phase samples of ≥1.5 by EIA. EIA identified 45 individuals as having a current measles virus infection, and CF identified 46 individuals; 42 infections were identified by both tests (Table 4). The serum

TABLE 2. Results of serum samples positive for antibody to measles virus by EIA, IFA, or HI and negative by PN[a]

Serum sample no.	Index	Titer		
	EIA	IFA	HI	PN
1	3.05	8	<4	<4
2	2.29	<8	4	<4
3	1.70[b]	<8	<4	<4
4	1.29	32	4	<4
5	1.21	8	4	<4
6	1.03	32	<4	<4
7	0.81	32	4	<4
8	0.56	32[b]	<4	<4

[a] Cutoff value for a positive result by EIA was an index of 1.0, that by IFA was a titer of 1:8 and that by HI and PN was a titer of 1:4.
[b] Positive results not confirmed by another method.

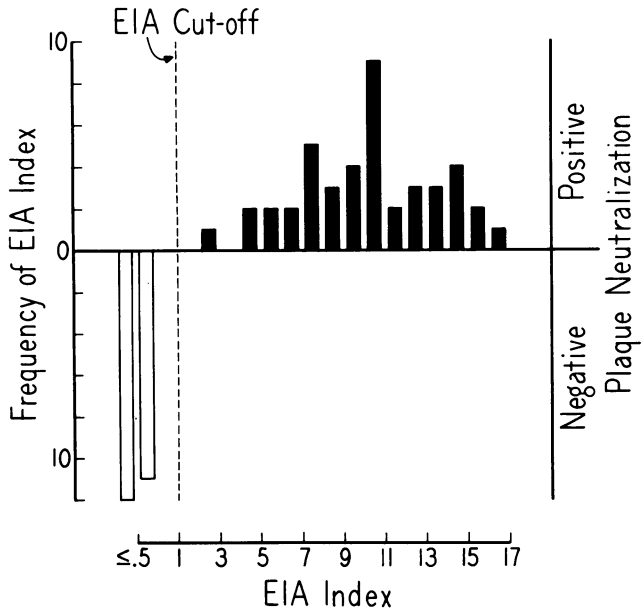

FIG. 2. Frequency of EIA indexes of the serum samples selected for study of immune status to varicella-zoster virus and correlation between EIA and PN results. Vertical dashed line (EIA cutoff) separates positive EIA results (index, ≥1.0) from negative EIA results (index, <1.0). Solid bars represent positive results by PN (titer, ≥1:4); open bars represent negative results by PN (titer, <1:4).

pair from one of the three cases missed by CF was unsatisfactory for testing by CF, another pair was taken too early to show antibody by CF, and one pair showed only a twofold increase in antibody titer. The indexes on the serum pairs from the four cases missed by EIA were high and stationary. On subsequent titration, one of the four CF-positive serum pairs showed a significant increase in antibody concentration by EIA (ratio, ≥1.5) at dilutions of 1:1,600 through 1:256,000. Titration of two of the four serum pairs did not result in higher ratios of their indexes at any dilution (1:100 through 1:6,400). Also, an increase in antibody concentration was not shown by EIA on separated IgG fractions of these sera (data not shown). The fourth serum pair misdiagnosed by EIA became contaminated and was unsatisfactory for further testing.

The insensitivity of CF in detecting antibody from past infections was shown by the absence of CF antibody in 55 of 174 serum pairs with standing antibody concentrations by EIA (Table 4) and by the lack of CF antibody in 59 of the 83 positive serum samples in the immunity status study (data not shown).

TABLE 3. Comparison of EIA and ACIF with PN for determination of immunity status to varicella-zoster virus infection

Test and result	No. of PN results		Predictive value of results (%)[a]	
	Positive	Negative	Negative	Positive
EIA				
Positive	43	0		100
Negative	0	23	100	
ACIF				
Positive	40	1		97.7
Negative	3	22	88.5	

[a] For formulas for predictive values, see Table 1, footnotes *a* to *c*.

TABLE 4. Comparison of CF and EIA for serodiagnosis of measles

Serodiagnosis by EIA (total no.)	No. with following serodiagnosis by CF:			
	Current infection[a]	Infection at some time[b]	Antibody not detected[c]	Unsatisfactory[d]
Current infection[a] (45)	42	1	1	1
Infection at some time[b] (174)	4	108	55	7
Antibody not detected[c] (32)	0	0	32	0
Unsatisfactory[d] (4)	0	2	0	2

[a] For serodiagnosis of current infection by EIA, the ratio of the index of the second serum sample to the index of the first serum sample was ≥1.5; by CF, there was a fourfold or greater increase in antibody titer between the first and second serum samples.

[b] Antibody was present, but there was not a significant increase in antibody concentration between the first and second samples of a pair.

[c] EIA index of the first and second samples was <1.0; CF titer of both samples was <1:8.

[d] EIA, Nonspecific reaction; CF, nonspecific reaction or anticomplementary.

(ii) Infection with varicella-zoster virus. By EIA, 19 individuals were identified as having a current infection with varicella-zoster virus, and 16 individuals were identified by CF. The serum pairs from two of the three cases missed by CF were unsatisfactory for testing by CF, and one pair showed only a twofold increase in antibody titer (1:8 to 1:16) (Table 5).

DISCUSSION

Although there are some reports on comparisons of EIA with other tests such as HI, CF, IFA (1, 2, 4, 9, 15), or fluorescent antibody to membrane antigen (2, 5) for detection of immunity status to measles or to varicella-zoster virus, only a rare report (4) is available on the correlation of EIA with PN results or on the evaluation of EIA for IgG antibody on paired serum samples for determination of current infection with these viruses.

In our laboratory, the routine test for determination of immunity status to measles virus is IFA, and that for testing immunity to varicella-zoster virus is ACIF. CF is our routine test for the serodiagnosis of current infection with either

TABLE 5. Comparison of CF and EIA for serodiagnosis of varicella-zoster virus infection

Serodiagnosis by EIA (total no.)	No. with following serodiagnosis by CF:			
	Current infection[a]	Infection at some time[b]	Antibody not detected[c]	Unsatisfactory[d]
Current infection[a] (19[e])	16	1	0	2
Infection at some time[b] (42)	0	22	19	1
Antibody not detected[c] (1)	0	0	1	0
Unsatisfactory[d] (0)	0	0	0	0

[a,b,c,d] See Table 4, footnotes *a* to *d*, for these designations.

[e] Of the 19 cases diagnosed by EIA, 14 were varicella, 4 were herpes zoster, and 1 was an inapparent infection after extensive exposure to a case of varicella. CF titers of pre- and postexposure samples in the latter case were 1:8 and 1:16, respectively.

virus. The described EIAs were developed and evaluated for use as alternate tests to these methods. EIA was the most sensitive of the various methods in detecting antibody to measles virus and scored as positive all those serum samples that were also positive by PN (Table 1 and Fig. 1).

However, EIA was no better than IFA and not as good as HI in identifying samples negative for neutralizing antibody to measles virus. Of the six samples, five that were scored positive by EIA but negative by PN were also scored positive either by IFA or HI or by both tests (Table 2). The results suggested that low levels of antibody were indeed present in these samples, but they may have had specificity for epitopes not important in neutralization. The antibody important in neutralization is thought to be directed against the hemagglutinin or the hemolysin of the measles virus or both (10, 11). The EIA antigen in the present study was a deoxycholate extract of infected cells cleared of intact virus by high-speed centrifugation and would therefore contain all of the proteins of the virion. The predictive value of a positive EIA result could probably be increased by the use of purified hemagglutinin and hemolysin antigen on the solid phase rather than a crude virus preparation. An alternate method would be the use of monoclonal antibodies on the solid phase for the capture of antigens of desired specificity from a subsequently added crude virus lysate as previously described (9).

Although the predictive value of a positive result was slightly higher for HI (95.3%) than for EIA and IFA (93.3%), the relative insensitivity of HI and IFA makes EIA the test of choice for determination of immunity status to measles virus. For purposes of specificity, our routine IFA test uses a starting serum dilution of 1:8 rather than 1:4, which contributes to its relative insensitivity.

ACIF was somewhat less sensitive than EIA in detecting antibody to varicella-zoster virus (predictive values of negative results were 88.5% by ACIF and 100% by EIA). The ability to score a positive PN result as positive was essentially the same for the two tests (predictive values of positive tests were 97.7% for ACIF and 100% for EIA). Overall, EIA was slightly better than ACIF, but not definitively so, since the sample of sera studied was relatively small (66 sera), and the differences in results between the two tests were not great (Table 3). These data are similar to those of a previous report (4) comparing PN with EIA performed in microtiter plates with step dilutions of the serum samples to an endpoint that was read visually. In that study, the predictive value of a negative reaction was 95.8%, and that of a positive reaction was 93.0%.

The number of negative sera (34.8%) in the sample studied was somewhat higher than would be expected from a normal population (16). The sera were from a group of teenagers whose history of chicken pox was negative or unknown and who were scheduled to be placed in an environment where several cases of chicken pox had occurred. Originally they were part of a larger group which was separated into two groups, those with a known history of chicken pox and those with a negative or unknown history. The removal of those individuals with a positive history of chicken pox and the inclusion of only those with a negative or an unknown history created a bias toward negative results (Table 3 and Fig. 2).

Results by EIA and CF for the serodiagnosis of measles were in agreement in 91% of the cases. CF missed three cases, and EIA missed four cases. One of the serum pairs misdiagnosed by EIA showed an increase in antibody concentration on further dilution of the samples. The lack of increase in antibody concentration between the samples of two of the pairs was not resolved by reassay at higher serum dilutions or by assay of IgG fractions free of IgM antibody. IgM antibody is reported to cause a prozone in some EIA titrations of IgG antibody and to cause a change in the slope of the titration curve (3). The ratios of the indexes of the IgG fractions of the two mentioned serum pairs were not significantly different from those of the whole sera and remained in a grey or equivocal zone of 1.3 to 1.4.

EIA was slightly better than CF in the serodiagnosis of varicella-zoster virus infection; 19 cases were diagnosed by EIA and 16 by CF. Of the 19 cases, 14 were varicella (chicken pox), and 4 were herpes zoster (shingles), and 1 was an adult who showed no symptoms but who had had extensive exposure to a child in the prodromal stage of varicella. A significant change in antibody concentration between pre- and postexposure serum samples was seen by EIA and ACIF but not by CF (EIA index of pre- and postexposure samples, 8.39 and 14.00, respectively; ratio, 1.67; pre- and postexposure sample titers by ACIF, 1:4 and 1:64, respectively, and by CF, 1:8 and 1:16, respectively).

EIA appears to be a satisfactory substitute for CF for the serodiagnosis of either measles or varicella-zoster virus infection. CF is technically more difficult and laborious than EIA and could be reserved for those cases that give equivocal results by EIA.

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