Antigen Requirements, Sensitivity, and Specificity of Enzyme Immunoassays for Measles and Rubella Viral Antibodies

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Received for publication 13 March 1979

Enzyme immunoassay (EIA) systems for measles virus and rubella virus were studied from the standpoints of requirements for suitable viral antigens and control antigens, and the sensitivity and specificity of the tests for detecting antibody elicited by past infection (determination of immunity status), and for serodiagnosis of current infections. Crude or semipurified measles virus antigens were satisfactory for EIA, but antigens derived by pelleting virus from infected cell culture fluids were slightly more specific in their reactivity than were antigens produced from lysates of infected cells. However, reliable rubella EIA antigens could be produced only from infected cell culture fluids, and they required density gradient purification to render them suitably specific. Even with gradient-purified rubella antigens, it was necessary to use antigen prepared in an identical fashion from uninfected cell culture fluids as a control on the specificity of reactions obtained with test sera. With appropriate viral antigens and control antigens, both measles and rubella EIA systems were highly sensitive and specific for determination of immunity status and for serodiagnosis of current infections. Antibody was detectable earlier in the course of infection by EIA than by hemagglutination inhibition or complement fixation, but this did not limit the diagnostic value of the test, since titer increases demonstrable by EIA were usually greater than those detectable by hemagglutination inhibition or complement fixation tests.

Enzyme immunoassays (EIA), as introduced by Engvall et al. (5, 6) in 1971 for quantitative determination of antigen or antibodies, have found wide application in a variety of viral systems. In most of these, antigen or antibody is adsorbed to a plastic surface, as described by Catt and Tregear (3) for radioimmunoassays. This not only facilitates performance of the test, but also provides uniform and stable concentrations of reagents for the assays. EIA possess the advantages of simplicity, sensitivity, stability, and safety of reagents and adaptability to automation. With the increased application of EIA to viral antibody assays, it has become apparent that there is a need for more reliable and potent antigens, for appropriate uninfected control antigens, and for improvement of the specificity of the test so that lower dilutions of serum can be assayed reliably for specific viral antibodies.

We report here on the behavior of viruses in two diverse families, namely, rubella (Togaviridae) and measles (Paramyxoviridae) viruses, in EIA for viral antibodies, on the development of suitable antigens and control antigens for each of the viruses, and on the sensitivity and speci-

ficity of EIA for serodiagnosis and determination of immunity status as compared with hemagglutination inhibition (HI) and complement fixation (CF) tests.

MATERIALS AND METHODS

Sera and cerebrospinal fluids studied. Paired serum specimens were examined from 23 patients with a clinical diagnosis of measles-like illness who showed ≥ 4 -fold measles virus antibody increases by HI and/or CF tests, and serum pairs were tested on 21 patients with clinical diagnoses of rubella-like illnesses who showed ≥ 4 -fold increases in rubella HI and/or CF antibody titers. Sera were examined from 33 individuals without clinical or serological evidence of measles virus infection and from 33 other individuals without evidence of a current rubella virus infection. Cerebrospinal fluids from 22 patients with a diagnosis of possible subacute sclerosing panencephalitis were examined for measles virus antibody.

EIA antigens. Measles virus antigens were produced from the LEC virus strain (1) propagated in roller bottle cultures of human fetal diploid lung cells. Cells were grown on Eagle minimal essential medium supplemented with 10% fetal bovine serum, and maintenance medium consisted of Eagle minimal essential medium with 2% fetal bovine serum. Cultures were

infected at a ratio of approximately 1 plaque-forming unit of virus to 5 cells; after 4 to 5 days of incubation at 36°C, the cultures showed an extensive cytopathic effect with many syncytia. The cells were dislodged into the fluid by shaking with glass beads, and the culture material was centrifuged at $700 \times g$ for 30 min at 4°C. The supernatant fluid was removed, and virus was concentrated by centrifuging at $78,000 \times g$ for 2 to 3 h and resuspending the pellet to 1/500 of the original volume in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-0.15 M NaCl buffer, pH 9.0, containing 0.002 M ethylenediaminetetraacetate. This constituted "cell-free" antigen, and the optimal concentration for sensitizing microtiter plates for EIA was found by block titration to be 1:1,000. This antigen dilution gave maximum reactivity with a positive antiserum and no color reaction with a negative serum, and the same dilution of uninfected control antigen showed no reactivity with positive or negative sera. The CF titers of the antigens were $\geq 1:512$. Antigen was prepared from the infected cells by resuspending the cells in the same volume of the above buffer, freezing and thawing three times, and then clarifying by centrifugation at $6,000 \times g$ for 30 min. Control antigens were prepared from uninfected cell cultures in the same manner. For the reasons discussed below, cell-free infected and uninfected antigens were adopted for routine measles EIA testing.

Rubella virus antigens were produced from the RV virus strain propagated in the BHK-21 line of baby hamster kidney cells. Cells in suspension were infected at a ratio of 1 plaque-forming unit of virus per cell and incubated at 37°C for 1 h with gentle shaking. The cells were then diluted appropriately in growth medium (Eagle minimal essential medium with 10% fetal bovine serum), and 9×10^7 to 10×10^7 cells were planted in roller bottles. After 48 h of incubation at 36°C, a complete monolayer formed, and the medium was replaced with maintenance medium consisting of Eagle minimal essential medium with 2% inactivated fetal bovine serum. After 18 to 24 h of incubation the cells were dislodged into the fluid with glass beads, the pH was adjusted to 8.5 by the addition of sodium bicarbonate, and the material was centrifuged at $6,000 \times g$ for 30 min at 4°C. The supernatant fluid was removed, and virus was pelleted by centrifugation at $78,000 \times g$ for 2 to 3 h and resuspended in 1/600 to 1/1,000 of the original volume in the Tris buffer described above. The concentrate was treated sonically briefly to disaggregate the virus and then centrifuged through a discontinuous gradient of 60 to 20% sucrose prepared in the Tris buffer described above. Centrifugation was performed at $110,000 \times g$ for 2 h in a no. 41 Spinco rotor. This is a modification of a method described by Bardeletti et al. (2) for rubella virus purification. The opalescent bands were collected through the bottom of the gradient tube, and corresponding fractions were collected from gradients prepared from uninfected BHK-21 cultures. To remove the sucrose, each fraction was diluted in Tris buffer and centrifuged at $78,000 \times g$ for 2 h, and the pellets were then resuspended to the original fraction volume in Tris buffer. Fractions were tested for hemagglutinating activity and for reactivity in EIA, and those with the highest specific viral activity were used in the EIA

for rubella antibody. The optimal dilution for EIA, as determined by block titrations, was 1:1,000. Hemagglutination titers of satisfactory antigens were $\geq 1:4,096$.

EIA. Tests were performed in MicroELISA substrate plates (Dynatech Laboratories, Alexandria, Va.) with round-bottom wells. The optimal dilution of rubella or measles antigen, as determined by preliminary block titrations against antisera of human origin and the same dilution of corresponding control antigen, was prepared in 0.006 M bicarbonate buffer, pH 9.5, and added to wells in a volume of 0.2 ml. The plates were held overnight at 4°C, after which unadsorbed antigen was removed by vacuum suction with a washer-aspirator (Dynatech Laboratories). Wells were washed once with 0.01 M phosphate-buffered saline, pH 7.3 (PBS), containing 0.05% Tween 20. To saturate protein adsorption sites, 5% bovine serum albumin in PBS was added in a volume of 0.35 ml, and plates were incubated for at least 4 h at room temperature. The fluids were then aspirated, and the wells were washed once with PBS-Tween 20 buffer. The antigen-sensitized plates could either be used immediately or stored at 4°C for several weeks.

An alkaline phosphatase conjugate was prepared from goat antiserum to human gamma globulin (Antibodies, Inc., Davis, Calif.) as previously described (7, 8). The optimal dilution for use in EIA, as determined by block titration with antigen-coated wells and varying dilutions of antisera, was found to be 1:2,000.

For the test proper, serial twofold dilutions of serum in PBS containing 1% bovine serum albumin, starting at 1:8, were prepared with 0.05-ml microdiluters in plates coated with viral antigen and with control antigen. For specificity controls on the conjugate, antigen and control antigen wells were incubated with diluent in lieu of test sera. PBS containing 1% bovine serum albumin in a volume of 0.05 ml was added to each well, and plates were incubated overnight at room temperature. Controls included known positive and negative sera and wells incubated with diluent instead of serum. The following day, the contents of the wells were aspirated and wells were washed three times with PBS. The optimal dilution of enzyme-labeled antibodies to human immunoglobulin G, prepared in 5% bovine albumin in PBS, was then added in a volume of 0.1 ml, and tests were incubated for 2 h at room temperature. After aspiration of the conjugate, wells were washed three times with PBS. The enzyme substrate consisting of *p*-nitrophenylphosphate (1 mg/ml) in diethanolamine (10%) buffer, pH 9.8, with 10^{-3} M $MgCl_2$ was added in a volume of 0.1 ml, and tests were incubated for 30 min at room temperature. The action of the enzyme was stopped with 0.05 ml of 3 N NaOH. Results were read by visual inspection against a white background. Antibody end points were taken as the highest serum dilution showing a visible color, and reactions were considered to be specific only if the corresponding wells containing uninfected control antigen showed no color. Antibody end points were reproducible between two different readers and between runs. Preliminary comparisons indicated that titers determined spectrophotometrically were generally twofold higher than those obtained by visual readings. Spectrophotometric readings were made with a Beckman DB spectrophotometer, using an optical density

at 405 nm of ≥ 0.06 , compared with a negative reading of ≤ 0.03 in the nonspecific control, as the antibody end point. The EIA titers reported in this study were based upon visual reading.

HI and CF tests. HI (4, 10) and CF (13) tests for measles and rubella viral antibodies were performed by standard microtiter procedures.

RESULTS

Development of EIA antigens. Little difficulty was encountered in producing adequately sensitive and specific EIA antigens for measles virus. Antigens derived either from lysates of virus-infected cells or from virus pelleted from infected culture fluids gave comparable antibody titers in preliminary experiments. However, test sera showed more reactivity with uninfected control antigens corresponding to the cell lysates than with those prepared from uninfected cell culture fluids. Therefore, cell-free viral antigens and control antigens prepared from culture fluids were adopted for further studies. Remarkably little nonspecific reactivity was seen in this measles EIA system. Of 108 sera, only 5 reacted with the control antigen at the 1:8 serum dilution. Two additional sera reacted to a titer of 1:32 with the measles control antigen, but these were shown to contain antinuclear antibody. In tests on cerebrospinal fluid, only 3 of 40 specimens showed reactivity at a 1:8 dilution against the measles control antigen.

In contrast to measles antigens, difficulty was encountered in preparing reliable rubella virus antigens for use in EIA. When unpurified preparations of various sorts were used, test sera reacted with control antigens to almost the same extent as with viral antigens, and efforts to improve the specificity of the test by using poly-*L*-lysine to attach antigen to the plate (14) were unsuccessful. Representative results are shown in Table 1.

Rubella virus preparations, and corresponding uninfected controls, which represented approximately 500-fold concentrates of either cells or culture fluids were subjected to density gradient centrifugation as described above, and the various fractions, at dilutions of 1:500, were assayed for activity in EIA against human reference sera. Table 2 shows the results of some representative experiments.

Importantly, it was found that human sera reacted with gradient fractions from uninfected control preparations which corresponded to the fractions containing the highest concentrations of viral activity. This was particularly marked in the case of cell-derived antigens, and less so with cell-free preparations. Fractions 5 and 6 from cell-free concentrates of rubella virus gave the highest antibody titers, and corresponding fractions from uninfected control material showed

TABLE 1. *Nonspecific reactivity in EIA with unpurified rubella antigens*

Antigen prepn	EIA titer of human antiserum in plates with antigen	
	Passively adsorbed	Coupled with poly- <i>L</i> -lysine
Virus-infected cells (500 $\times$) sonically treated	512	1,024
Control uninfected cells sonically treated	256	1,024
Cell-free virus concentrated 500 $\times$ by high-speed centrifugation	2,048	8,196
Control cell culture fluids concentrated 500 $\times$ by high-speed centrifugation	512	2,048
Rubella CF antigen ^a	64	256
Control CF antigen	32	256
Rubella hemagglutinating antigen ^a	64	128
Uninfected antigen prepared in same manner as hemagglutinating antigen	32	128

^a Reagent used for standard serological test.

TABLE 2. *Specific and nonspecific activity of rubella EIA antigens purified by density gradient centrifugation*

Gradient fraction no. (diluted 1:500)	EIA titer of human antiserum against fractions derived from:			
	Cell-free rubella virus (500 $\times$)	Cell-free control fluid (500 $\times$)	Rubella-infected cell lysate (500 $\times$)	Uninfected cell lysate (500 $\times$)
1	0 ^a	0	0	0
2	0	0	0	0
3	16	16	64	64
4	512	8	256	256
5	2,048	8	256	512
6	2,048	32	256	256
7	1,024	512	512	512
8	1,024	512	512	512

^a Titer <1:8.

minimal reactivity with human sera. Thus, these materials were used for virus-specific and control antigens in the rubella EIA system. Block titrations showed the optimal dilution of viral antigen for use in EIA to be 1:1,000, and at this dilution of control antigen relatively little reactivity was seen with test sera. Of 104 sera, 47 had titers of 1:8 with the control antigen, and 14 had titers of 1:16; two additional sera with antinuclear antibody had titers of 1:64 with the control antigen.

Sensitivity and specificity of EIA for determination of immunity status to measles and rubella virus infections. The scattergrams in Fig. 1 compare measles and rubella titers obtained by EIA to those obtained by HI and CF for individuals without evidence of a current measles or rubella virus infection. There was excellent correlation between presence or absence of antibody by HI and by EIA. EIA titers were higher than HI titers; in the case of rubella they tended to be 2- to 8-fold higher, and in the case of measles the spread was greater, with EIA titers generally in the range of 2- to 32-fold higher than HI titers. The low sensitivity of the CF test for detecting antibody elicited by past infection is reflected by the large number of sera with CF titers $\leq 1:8$ and markedly higher EIA titers.

Sensitivity of EIA for serodiagnosis of

measles and rubella virus infections. Figure 2 shows scattergrams in which acute- and convalescent-phase EIA titers are compared to HI and CF titers in individuals with current measles or rubella virus infections. All of the sera in which antibody was demonstrable by EIA but not by HI or CF were acute-phase sera, indicating the greater sensitivity of EIA for detecting low levels of antibody at the beginning of the serological response to infection.

Although convalescent-phase antibody titers demonstrated by EIA were usually in the range of 1:512 to 1:8,192 for both viruses, there were some differences in the antibody responses by HI and CF to the two viruses, which are evident from the different patterns in the scattergrams. In the case of rubella virus, convalescent-phase HI titers were generally $\geq 1:128$, and EIA titers tended to be 2- to 16-fold higher. In the measles

FIG. 1. Sensitivity of EIA for detecting measles and rubella antibodies in individuals without evidence of current infection.

FIG. 2. Sensitivity of EIA for detecting measles and rubella antibodies in acute (○)- and convalescent (●)-phase sera from individuals with current infections.

patients, a number of the convalescent-phase HI titers were $\leq 1:128$, and EIA titers were 2- to 64-fold higher. In contrast to HI titers, the convalescent-phase CF titers for measles virus were generally $\geq 1:128$; EIA titers were 2- to 32-fold higher. In rubella virus infections, on the other hand, convalescent-phase CF antibody titers were $\leq 1:64$, and EIA titers were generally in the range of 128- to 1,024-fold higher.

A major concern when a new serological technique shows increased sensitivity with frequent ability to demonstrate high antibody levels in acute-phase sera is the possibility that there may be a diminished ability to demonstrate significant increases in antibody titer between acute- and convalescent-phase sera. However, the data presented in Tables 3 and 4 show that EIA usually demonstrated markedly greater in-

creases in antibody titer than were demonstrable by HI and CF.

Figure 3 compares measles virus EIA titers with HI and CF titers on cerebrospinal fluid from patients with suspected subacute sclerosing panencephalitis with a measles virus etiology. Again, there was excellent correlation between presence or absence of antibody by EIA and by the other methods, and EIA titers were markedly higher. It is noteworthy that EIA titers of 1:32 or greater were obtained on the few sera with low HI or CF titers of 1:2 or 1:4 that would be considered equivocal and would be difficult to interpret.

DISCUSSION

These investigations have demonstrated that the feasibility of EIA and the specificity of the

TABLE 3. Comparison of the magnitude of antibody titer rises shown by EIA and by HI and CF in measles virus infections

Titer rise by EIA	No. of patients	No. of patients showing indicated titer rise													
		Measles HI							Measles CF						
		2×	4×	8×	16×	32×	64×	≥128×	2×	4×	8×	16×	32×	64×	
2×	1	1								1					
4×	0														
8×	3	3								2	1				
16×	5	1		1	1	1		1	1	1	2	1			
32×	3 ^a					3						1	1		
64×	1							1				1			
128×	3	1			1	1						2	1		
256×	3		3								1		2		
512×	1		1											1	
1,024×	3			1			1	1						3	

^a One patient showed anti-complementary activity in CF test.

TABLE 4. Comparison of the magnitude of antibody titer rises shown by EIA and by HI and CF in rubella virus infections

Titer rise by EIA	No. of patients	No. of patients showing indicated titer rise											
		Rubella HI						Rubella CF					
		2×	4×	8×	16×	32×	64×	128×	256×	2×	4×	8×	16×
2×	1			1									1
4×	0												
8×	1						1					1	
16×	1			1							1		
32×	4		1			2	1			1	3		
64×	7			1	1	1	2	1	1	3	2	1	1
128×	5				1	3			1	1	3	1	
256×	1							1			1		
512×	0												
1,024×	1								1			1

FIG. 3. Sensitivity of EIA for detecting measles virus antibodies in cerebrospinal fluid.

system can vary greatly with different viruses and that optimal conditions for antigen production must be individually defined for each virus to be tested. Several types of crude or partially purified measles virus antigens were suitable for

use in EIA, but those free from cellular debris were somewhat more specific in their reactivity. On the other hand, preparation of reliable and specific rubella virus antigens required purification by density gradient centrifugation and careful attention to selecting the fractions for which corresponding gradient fractions from uninfected control material showed no nonspecific reactivity. Again, antigens prepared from cell-free virus were markedly more specific than were those derived from cell lysates. Early reports on the use of EIA for assay of rubella antibody indicated that crude commercially available antigen preparations could be used satisfactorily in the system (16, 17). However, our findings were in accord with later reports (9, 12) that reliable rubella antigens required purification by density gradient centrifugation. Once purified, however, the antigens are usable at dilutions roughly corresponding to the original volume of cell culture fluid used for concentration and purification,

and thus their use is not prohibitively expensive.

Some of the EIA procedures which have been described for detection of viral antibody do not include the use of uninfected control antigens. However, it is becoming increasingly apparent that this is essential as a control on the specificity of the reactivity of each test serum. An occasional serum will react to as high a titer with uninfected control antigen as with the specific viral antigen, particularly sera containing anti-nuclear antibody. Our results have shown that even when density gradient-purified rubella antigens are used, uninfected control antigens are essential, since certain gradient fractions from uninfected BHK-21 cell culture fluids processed in the same manner as the rubella antigen showed nonspecific reactivity with human sera in the EIA system.

Through the use of antigens of maximum specificity, it was usually possible to obtain reliable EIA results at serum dilutions as low as 1:8, and with few exceptions at dilutions of 1:16. This is in contrast to certain other EIA systems described for viral antibody assay in which it has been necessary to use a starting serum dilution of 1:50 or 1:100 to avoid nonspecific reactivity. The use of an enzyme conjugate prepared from antibodies purified by immunoadsorption (8), and the use of a bovine serum albumin solution to saturate protein adsorption sites after sensitization of the plates with antigen, have also contributed to the specificity of our EIA system. When measured spectrophotometrically (7) the ratios obtained by dividing the optical density with viral antigen by that with control antigen were in the range of 10 to 20 for optimal dilutions of positive sera. This is much higher than such ratios reported in certain other viral EIA systems (15) and further indicates the marked specificity of the EIA systems reported here.

The sensitivity of the EIA for measles and rubella virus antibodies was such that equivocal low titers which would cause difficulty in interpretation were rarely seen in sera from past infections, making the test particularly useful for determining immunity status to these viruses. EIA avoids the problems which might be caused in the HI test by incomplete removal of nonspecific inhibitors and those relating to the lack of sensitivity of the CF test for detection of antibodies from past infections. When low CF or HI titers which would be difficult to interpret were obtained in testing cerebrospinal fluid for measles antibody, the corresponding EIA titers were clearly positive at 1:32 or greater.

The ability to detect viral antibody early in the course of infection did not interfere with the diagnostic value of EIA, and, in fact, the poten-

tial for early antibody detection offers promise that EIA can provide a more rapid serodiagnosis of viral infections, as has been suggested by other workers (11).

With the availability of adequately sensitive and specific antigens, and with the use of appropriate control antigens, EIA for measles and rubella virus antibodies could become a rapid and reliable method for determination of immunity status and for serodiagnosis of infection in a clinical setting.

ACKNOWLEDGMENTS

We are indebted to David Cottam for assistance in these studies.

This study was supported by Public Health Service grant AI-01475 from the National Institute of Allergy and Infectious Diseases.

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