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Respiratory Syncytial Virus Serology by a Simplified Enzyme-Linked Immunosorbent Assay

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A simplified enzyme-linked immunosorbent assay (ELISA) which utilized commercially available reagents was developed for respiratory syncytial virus (RSV)-specific immunoglobulin G. An analysis of the inherent variation of the assay allowed the setting of strict criteria for determining a significant change in RSV antibodies. The ELISA was more sensitive than the standard complement fixation or microneutralization tests in a carefully studied group of 32 RSV-infected adults. The ELISA correlated closely with complement fixation serological testing in 25 patients. The use of purified antigens might allow the development of a more sensitive ELISA.

Serological investigation of respiratory syncytial virus (RSV) infection can be unreliable when performed by complement fixation (CF), especially on infants. Other serological tests, such as the plaque reduction assay or neutralization test, require tissue culture expertise and are laborious to perform. The latter tests have not become widely available, and most centers rely on the CF test.

The enzyme-linked immunosorbent assay (ELISA) has been adapted for use in many diagnostic tests, including serological investigations of viral infections (8). In addition to a high level of sensitivity which may allow the use of serology to diagnose infection within days rather than the traditional 2 or 3 weeks (6), the ELISA is a reproducible and objective test which can be performed on small amounts of serum.

An ELISA method for RSV serology which appears to be sensitive has been reported (7). The method involved the use of RSV-infected cells as the antigen and fourfold dilutions of serum to determine endpoint titers. We report a simplified ELISA for RSV antibodies which utilizes commercially available antigens and reagents and requires only a single dilution of serum, when a ratio of the ELISA results is used.

MATERIALS AND METHODS

Antigen. Commercial RSV CF antigens (Microbiological Associates, Bethesda, Md.) were tested for activity against our standard serum (H-47/77, a serum with a high RSV titer by neutralizing and complement fixation testing), and we obtained lots which appeared to have high activity when compared with control antigens from the same source.

Enzyme-antibody conjugate. Antiserum raised in goats against human immunoglobulin G (IgG) and rendered γ-chain-specific by absorption (Antibodies Inc., Davis, Calif.) was precipitated with ammonium sulfate to obtain the globulin fraction. This fraction was conjugated with alkaline phosphatase (Sigma type VII) by the method of Engvall and Perlman (3). A commercial alkaline phosphatase-porcine antihuman IgG conjugate (Orion Diagnostica, Medical Technology Corp., Hackensack, N.J.) was also found to be satisfactory.

ELISA serology. The technique of Voller et al. (8) was modified for this ELISA serology for RSV-specific IgG. Optimal volumes and dilutions of reagents were determined by checkerboard titration.

Plastic microtiter plates (Microelisa, Cooke Laboratory Products, Alexander, Va.) were coated with 0.1 ml of a 1:100 dilution of the RSV antigen in carbonate-bicarbonate buffer (pH 9.6) (8). The plates were stored for at least 18 h or up to 2 months at 4°C. Before use, each well was washed three times with 0.2 ml of phosphate-buffered saline (PBS) buffer with 0.05% Tween 20 (PBS-Tween). The antigen-coated wells were filled with 0.1 ml of dilutions of the sera to be tested. The sera to be tested and the conjugate were diluted in PBS-Tween with 1% fetal calf serum. The sera were allowed to react for 18 h at room temperature.

After washing three times with PBS-Tween, 0.1 ml of the enzyme conjugate diluted 1:200 was added to the wells, and the plates were incubated at 37°C for 2 h.

After washing with PBS-Tween, 0.2 ml of the substrate solution was added to each well. The substrate was *p*-nitrophenylphosphate (Sigma 104) in 10% diethanolamine buffer (8). After 45 min at room temperature, the reaction was stopped with 0.05 ml of 3 M NaOH. The absorbance of the final solution was read at 400 nm in a Beckman spectrophotometer.

Each dilution of serum was run in duplicate. If the paired wells were of different optical density by visual inspection, or if their absorbances varied by >20%, the sample was retested. The standard serum was run on every plate to enable standardization of results obtained with different plate lots and on different dates.

CF (1) and microneutralization (4) tests were done by standard methods.

Sera. Paired serum samples were obtained from two groups of subjects. Group 1 consisted of 32 volunteers who were inoculated with RSV and followed for 7 to 10 days with daily clinical examination and culture of nasal secretions (5). Those volunteers who were ill with respiratory signs and had RSV cultured were regarded as infected (true positives). Group 2 was 25 patients from whom paired sera were obtained during an outbreak of RSV in a nursing home. The convalescent sera were obtained at least 2 weeks after the acute serum in every case. In addition, paired sera from eight patients with other viral infections were tested to ensure specificity of the ELISA test.

Interpretation. The ELISA titer (E titer) was defined as the highest serum dilution which resulted in an absorbance which was greater than twice the background level (absorbance of antigen-coated wells without serum). Inspection of the absorbance data at dilutions from 1:50 to 1:3,200 demonstrated that an increase in absorbance at a dilution of 1:100 correlated with an increase in endpoint titer. This is demonstrated graphically in Fig. 1 and 2. To determine valid criteria for a significant rise in the absorbance value in this ELISA, the standard serum was run 50 times. The

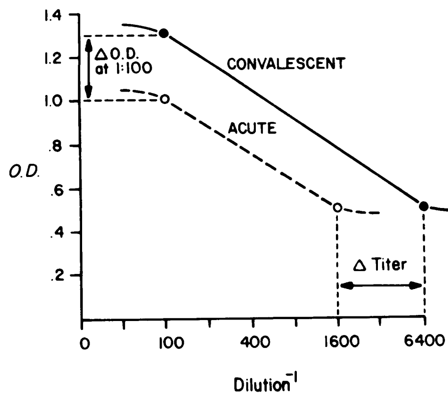

FIG. 1. *Relationship of ELISA endpoint titers with absorbance changes (optical density) at one titer is demonstrated in positive paired sera from a patient with RSV infection (E ratio = 1.32).*

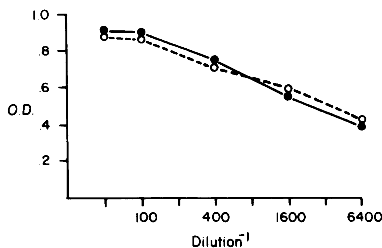

FIG. 2. *Relationship of ELISA endpoint titers with absorbance changes (optical density) at one titer is demonstrated in negative paired sera from a patient without RSV infection (E ratio = 1.04).*

absorbance values at dilutions from 1:50 to 1:6,400 had a maximal standard deviation of 7% of the mean value. An interval of three standard deviations (±21% of the mean) would therefore have a very high probability of including every absorbance value of a single dilution of a serum (assuming a normal distribution of absorbance values about the mean). Conversely, a convalescent serum which had a mean absorbance of >121% (standard deviation, >3) of the cognate acute serum has a very high probability (>99.9% from the table of area of the standard normal curve) of representing a true increase in specific antibodies. We defined a significant increase in absorbance as an E ratio: absorbance of convalescent serum at 1:100/absorbance of acute serum at 1:100 = ≥1.22. An E ratio of ≥1.22 was considered a positive serological result. Variation of absorbance values among test runs is controlled by use of the E ratio method. Since all sera were run in pairs on the same plate and compared by ratio, this value will remain essentially the same in different runs.

RESULTS

Table 1 displays the results of both ELISA methods of determining changes in RSV antibodies compared to culture evidence of infection in 12 volunteers in group 1 who were inoculated with RSV. The E ratios in these 12 pairs of sera demonstrated only slight variation when run on three separate occasions. In no case did the variation change the serological results from positive to negative or vice versa.

Table 2 shows the results of CF and microneutralization tests and E ratio determinations in 32 subjects in relation to their shedding of RSV. Table 3 compares the CF and microneutralization results and E ratio determinations in 25 sera collected during an outbreak of RSV disease. (Specimens for viral isolation were not obtained from all of these subjects.)

Sera from eight patients with other viral infections were also tested to ensure specificity of the test. These patients had influenza A, influenza B, herpes simplex, or rhinovirus isolated

TABLE 1. *Comparison of two ELISA serological methods and culture data*

Subject	Culture	E titer (fold rise)	E ratio
1	+	>4	1.36
2	−	0	1.04
3	−	0	1.07
4	−	0	1.06
5	+	>4	1.52
6	+	>4	1.29
7	−	0	0.95
8	+	>4	1.50
9	−	0	1.04
10	+	0	1.10
11	+	>4	1.57
12	+	2	1.29

TABLE 2. *Serological results in group 1 compared with culture data*

Serological test	Culture	
	Positive	Negative
CF		
Positive[a]	5	0
Negative	4	23
MN[b]		
Positive[a]	4	0
Negative	5	23
E ratio		
Positive[c]	6	0
Negative	3	23

[a] A greater than fourfold titer rise.
[b] MN, Microneutralization.
[c] Greater than 1.22.

TABLE 3. *Comparison of serology in group 2*

Culture	CF	MN[a]	E ratio
Positive	7[b]	2[b]	7[c]
Negative	18	23	18

[a] MN, Microneutralization.
[b] A greater than fourfold titer rise.
[c] Greater than 1.22.

TABLE 4. *Comparison of the indices of the serological methods in group 1*

Indices	CF (%)	MN[a] (%)	E ratio (%)
Sensitivity	55	44	67
Specificity	100	100	100
Predictive value of positive serology	100	100	100
Predictive value of negative serology	85	82	88
Efficacy[b]	88	85	91

[a] MN, Microneutralization.
[b] Defined as (true positive + true negative)/all tests.

from their nasal secretions. These patients all had "negative ratios" with mean E ratio of 1.00 (±0.03) by this RSV ELISA, thus confirming specificity.

DISCUSSION

Although the number of tests performed was not large enough to draw firm conclusions regarding the relative merits of these serological methods, these early data demonstrate that in the two groups surveyed the simplified ELISA was an acceptable serological test. Table 4 presents the indices of these tests in the 32 volunteers in group 1 in whom true positives and true negatives are clearly defined, for the purpose of comparison. It is interesting to note that even with the use of similar crude antigens (commercial CF antigens), the ELISA was somewhat more sensitive than the CF. The ELISA was certainly easier than the CF to perform, and with the elimination of serial dilutions, became even more so. Since the ELISA results are numerical, greater objectivity and reliability are possible than with CF or neutralization tests. The numerical ELISA results also allow the determination of criteria for significant change based on statistical probability reasoning.

The use of the E ratio or of a change in absorbance in the ELISA as an indication of a change in titer of RSV-specific antibodies might be questioned. ELISA measures the interaction between antigen and antibody, and the results are affected by both the concentration and avidity of antibodies. A change in either factor will be reflected in a change in the absorbance (2). However, a meaningful increase in a serum pair of the number of specific antibody molecules or their avidity is probably due to exposure to the antigen of interest. Therefore, a statistically significant change in the E ratio, whatever the mechanism, is likely to correlate with exposure to the antigen. It is important, of course, to pick a dilution of serum at which most subjects will not have enough antibody in the acute serum to saturate antigen sites in the well. For our subjects and the conditions of this assay, the appropriate dilution was 1:100; other assays and populations will require a separate analysis to determine the optimal testing dilution. We feel the use of statistically defined E ratios, rather than more arbitrary definitions of seropositivity (2× background) or seroconversion (fourfold rise in endpoint titer), would enhance the usefulness of ELISA serological methods.

The use of more specific RSV antigens in a similar ELISA might improve the sensitivity of the assay. This laboratory is collaborating in the search for and testing of specific RSV antigens. The identification of such antigens will allow the development of an efficient serological test for all age groups. This ELISA could be adapted with such antigens to examine the responses of all classes of immunoglobulins in primary and repeat infections.

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