Enzyme-Linked Immunosorbent Assay for Detection of Respiratory Syncytial Virus Infection: Application to Clinical Samples

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An enzyme-linked immunosorbent assay (ELISA) for respiratory syncytial virus antigens was applied to the rapid diagnosis of acute infections in children and was compared with viral culture and immunofluorescence tests. The ELISA test employed commercially available reagents and was run on a day-to-day basis as specimens were received in the laboratory. Sensitivity and specificity by ELISA were 82 and 95%, respectively, compared with culture. In the same specimens, the sensitivity and specificity by immunofluorescence were 86 and 96%, respectively. Nasopharyngeal aspirates were proven to be a better source of viral antigen than were nasopharyngeal swabs. ELISA-positive samples remained positive even when left unrefrigerated for a week or mailed to the laboratory in plastic containers. Respiratory syncytial virus ELISA, like culture, became negative as the disease progressed and showed no superiority over culture for diagnosis late in the illness.

Recently, enzyme immunoassays have been applied to the detection of viral antigens in respiratory secretions by a number of laboratories (1, 2, 5, 6, 8, 9). We recently developed an enzyme-linked immunosorbent assay (ELISA) detection system for respiratory syncytial virus (RSV) antigens, which employed commercially available reagents (5). The system was refined so that it detected 50 PFU of virus per ml and 10 ng of partially purified antigen per ml. The clinical investigation reported here was designed to examine how well this test performed under day-to-day use in a diagnostic laboratory during the winter of 1980 and 1981, to compare its sensitivity and specificity with traditional tissue culture virus isolation techniques and rapid diagnosis by immunofluorescence (IF), and to test certain hypotheses concerning the obtaining and handling of specimens for antigen detection.

MATERIALS AND METHODS

Patients and specimens. All respiratory secretion specimens received in the Diagnostic Virology Laboratory of the Children’s Hospital Medical Center, Boston, Mass., during the period of December 15, 1980, to March 31, 1981, were examined by ELISA, by IF, and by tissue culture inoculation. The patients were for the most part hospital inpatients and ranged in age from <1 month to about 12 years, with 83% of them under 1 year of age. Specimens were taken at the discretion of the house staff on admission, shortly thereafter, or, in the case of nosocomially acquired infections, at the onset of symptoms. If serial specimens were sought, permission was first obtained from the parents, and all subsequent samples were taken by a specially trained research nurse.

Nasopharyngeal (NP) aspirates were obtained by suction from the nasopharynx as described previously (7). Some aspirates from intubated patients were of tracheal, rather than NP, secretions. For comparison with NP aspirates, some NP swab specimens were also obtained. A flexible wire swab (Calgiswab) was placed in the nasopharynx, left in place for 30 s, and then removed and cut off in 1 ml of phosphate-buffered saline (pH 7.2) containing 0.5% gelatin and antibiotics. Immediately after this, an NP aspirate was obtained in the usual fashion by gentle suction through a no. 5 or a no. 8 French catheter. Specimens were undiluted when brought to the laboratory, and were often found in the tubing of the suction catheter or in the plastic trap or both. Phosphate-buffered saline (0.5 to 1.0 ml) was usually added to the specimen to facilitate its removal into a glass vial for further study. It was our policy to accept any specimen with grossly visible secretion present even though the volume might be very small.

Viral culture. For recovery of RSV and other viruses in cell culture, 0.05 ml of NP secretion was inoculated into duplicate roller tubes of HEP-2 (Flow Laboratories, Inc.), primary rhesus monkey kidney (Microbiological Associates), and diploid human foreskin fibroblast cells (strain 350Q, passages 14 though 25, obtained from John Zaia). Tubes were incubated, either stationary at 36°C or on a roller drum at 34°C (strain 350Q only), and examined at 2- to 3-day intervals for cytopathic effect. RSV was identified by its
characteristic cytopathic effect and, in most instances, by indirect IF with rabbit RSV antiserum.

Protein measurements in some secretions were determined as described in the report of the development of this test (5).

RSV ELISA. The RSV ELISA was performed, in general, as described (5). For the first incubation of NP secretions, 0.05 ml of secretion and 0.05 ml of 20% N-acetylcysteine (Mucomyst) were mixed in each well and incubated overnight at room temperature or over the weekend at 4°C. On each microtiter plate six wells were used for replicate samples of a control NP secretion containing 4 mg of total protein per ml, obtained from an adult who was not infected with RSV at the time. All of the secretions were tested in duplicate. A positive ELISA reading was considered to be one in which the average of the duplicate wells was greater than three standard deviations above the mean of the six negative control wells in that plate. Pilot studies showed that the incubation of secretions with capture antibody could be carried out at 4°C over a weekend (rather than at room temperature overnight) without sacrificing either sensitivity or specificity, and this protocol was used for specimens which arrived in the laboratory and were tested on Fridays.

The suitability of the control NP secretion was confirmed by a statistical comparison of replicate control wells with wells containing negative secretions from infants sampled both during and immediately after the RSV outbreak and tested as part of the routine diagnostic work reported below. The mean and variance of the optical densities from replicate control wells did not differ significantly from those of negative (i.e., not showing RSV by culture, IF, or ELISA) specimens in any of the four plates so analyzed (P > 0.05, F test). A total of 31 negative secretions, tested in duplicate, were analyzed from four different tests.

To confirm the specificity of the reaction with certain specimens, blocking tests were performed with guinea pig serum raised against partially purified RSV (Long strain). Greater than 60% reduction of the mean net absorbance by this antiserum was considered to be evidence for specificity.

IF. The methods of Gardner and McQuillen were used for IF testing (3). Antiserum to RSV was raised in rabbits by multiple injections of HEp-2-grown RSV (Long strain) and was extensively absorbed with HEp-2 cells before use. Sheep anti-rabbit fluorescein conjugate was purchased from Burroughs Wellcome Co. The preparation of NP epithelial cells and fixation to slides were as described previously (3). Slides were read by one of two investigators, using a Zeiss epi-fluorescence microscope with a 40× oil immersion objective.

Statistical methods. The two-tailed Student t test and chi-square tests without Yates’ correction were used to test significant differences.

RESULTS

Comparison of ELISA with culture and IF. Table 1 shows a three-way comparison of culture, IF, and ELISA for detection of RSV in NP aspirates. Both IF and ELISA gave false-positive and false-negative results, frequently without agreement on the same specimen. The sensitivity and specificity of the two rapid methods in relation to culture were similar—82 and 95% for ELISA, 86 and 96% for IF. In turn, the sensitivity and specificity of ELISA in relation to IF were 84 and 90%, and those of IF in relation to ELISA were 87 and 89%.

Specimens yielding either false-positive or false-negative readings were the objects of further studies. False-positive IF results could be confirmed without exception by staining of duplicate slides and blind reading by a second observer.

Attempts were made to block the ELISA reaction in the four NP aspirates which were ELISA positive but negative by both IF and culture. Blocking was successful in one of the four, one was not available in adequate quantity, and two tested negative in a repeat examination.

We considered that specimens falsely negative by ELISA might have been excessively diluted during processing because of the small volume of secretion obtained. To investigate this, we measured total protein in 13 ELISA false-negatives and compared the results with a random sample of true-positives and true-negatives. The mean concentrations (milligrams per milliliter) were 0.84 ± 0.62 (standard deviation), 1.07 ± 0.63, and 1.29 ± 0.64, respectively. These were not significantly different from each other. There was, however, a cluster of three samples with very low or undetectable protein concentrations in the false-negative group, an indication of possible inadequate material for testing.

If false-negativity by ELISA was related to low virus titer, some relationship might be found with the number of days required for those specimens to produce a cytopathic effect in cell culture. Specimens positive by both ELISA and culture were detected in culture in a mean of 8 days (±7), whereas those positive only in culture were detected in 13 days (±7). This difference is suggestive but not significant.

We also subjected 17 false-negative and 7 true-positive specimens to sonication with a probe sonicator to discover whether antigen

<table>
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<th>TABLE 1. Comparison of culture, IF, and ELISA for the detection of RSV in NP aspirates from infants with acute respiratory disease</th>
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<td><strong>Specimens were:</strong></td>
</tr>
<tr>
<td>ELISA positive</td>
</tr>
<tr>
<td>ELISA negative</td>
</tr>
<tr>
<td>IF positive</td>
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<tr>
<td>IF negative</td>
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might be freed or dispersed by this technique. Although the optical density of previously positive specimens was increased by an average of 40% (±22%) only one false-negative specimen converted to positive.

We considered the possibility that a false-negative ELISA might be a reflection of antigenic heterogeneity, and the bovine antiserum to bovine RSV might not cross-react with some strains which stained with rabbit anti-Long strain or which grew in tissue culture. This possibility was tested in two ways. First, samples negative by ELISA but positive by IF were retested by IF but incubated with the bovine anti-bovine RSV used in the ELISA test and then stained with a fluoresceinated antiserum to bovine immunoglobulin (Burroughs Wellcome Co.). All of the samples tested in this way were stained identically with the bovine serum and the rabbit serum. Seven samples for which replicate slides were not available were retrieved as HEP-2 or 35Q passages from the freezer and tested directly by ELISA. Five of seven samples produced positive readings when tested in this way. Attempts to recover and passage in tissue culture the remaining two samples were not successful.

Finally, we considered whether inconsistent results might be more frequent in older children who might be undergoing reinfection and therefore excreting smaller amounts of virus or mixtures of virus and antibody. Although false-negative ELISAs were more frequent in the group one year of age and older (40%), this did not prove to be a significant difference from the group as a whole.

Comparison of swabs and aspirates as specimens for ELISA. Most viral respiratory cultures are obtained with either a throat or a nasopharyngeal swab. We have already shown that the carrying medium we use routinely has a marked dampening effect on the ELISA test (5). We further investigated this problem by examining the use of NP swabs immersed in a carrying medium known not to be inhibitory in our test. The results are shown in Fig. 1. Nine infants who were excreting RSV were sampled as described above, first by NP swab and then by NP aspirate. Six of the nine aspirates were ELISA positive, compared with only one ELISA positive by swab. Tissue culture tests were positive in eight and three infants, respectively (data not shown).

Mistreatment of samples. Since transportation of samples for virus detection is often a problem due to centralization of most diagnostic facilities, we examined the stability of RSV antigens and the infectivity in aspirates obtained from children known to be RSV positive. The samples were received in the laboratory on ice and then separated in portions which were cultured; frozen at −70°C immediately; placed in a vial on a bench top from which small amounts were removed at 3, 5, and 7 days for culture and freezing; or placed in a small plastic tube, wrapped carefully, and mailed to the laboratory, using a mail box outside the laboratory building. When the mailed samples were received again in the laboratory, usually after 5 days, a portion was immediately cultured, and the remainder was frozen. Finally, all of the frozen samples were thawed and tested by ELISA. Results are shown in Table 2.

ELISA tests remained almost uniformly positive under these conditions. Cultures, in contrast, became negative in the majority of the mistreated samples.

Specimens obtained late in infection. We obtained serial specimens from a group of 27 infants whose initial samples grew RSV or were positive by IF or by ELISA to discover how rapidly antigen became undetectable as recovery proceeded. The results of these studies are shown in Fig. 2. Because the numbers were small, particularly in later samples, it is difficult
TABLE 2. Mistreatment of NP aspirate specimens: effect of storage on a bench top or mailing without ice on culture and ELISA results

<table>
<thead>
<tr>
<th>Method of virus detection</th>
<th>No. of NP aspirates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. positive/total no. tested after:</th>
<th>Storage on bench top for:</th>
<th>Mailing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>8</td>
<td>4/8/2/6</td>
<td>3–5 days</td>
<td>2/8</td>
</tr>
<tr>
<td>ELISA</td>
<td>6</td>
<td>5/5/5</td>
<td>5/5</td>
<td>5/5</td>
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<sup>a</sup> All aspirates were positive at time zero by both methods.

to interpret these data, but it appears that the sensitivity of ELISA was less than that of culture from the very first samples obtained and remained so throughout the course of the infection. The paradoxical reversal of this pattern on days 3 and 4 is unexplained.

DISCUSSION

A number of groups, including our own, have previously described ELISA tests for the detection of viral antigens in respiratory secretions (1, 2, 5, 6, 8, 9). The clinical sensitivity of the test reported here, 82%, is lower than what we wished, but still high enough to be useful as a rapid test. It is very similar to the 79% reported by Chao et al. (2), somewhat higher than the competition ELISA of Hornsleth et al. (6), and lower than the test reported by Sarkkinen et al. (9). It is, however, not possible to compare such results in any meaningful way since there is no uniformity in the samples tested. Our result was achieved with an "on-line" test, which was done three to five times a week, and the results were available to the clinician usually within 24 h. Moreover, we made it a policy to accept specimens as long as there was visible secretion in the tubing submitted.

The reason for our 18% false-negative results is not entirely clear. Some samples were probably too diluted. Others may have had only small amounts of free antigen present because of the age of the patient or the timing of the sample. Our studies indicated that there were other possible reasons which were probably not valid. There were no false-negatives attributable to our use of an anti-bovine RSV antiserum. Sonication with a probe sonicator increased the optical density of previously positive specimens, but converted only 1 of 17 ELISA false-negatives to positive. Sarkkinen reported that sonication often increased absorbance by secretion samples by twofold (9). It appears possible that this degree of change with sonication might be due to their use of antinucleoprotein antisera, necessitating the rupture of cells for release of detectable antigen.

Aspirates were clearly better sources of antigen than nasopharyngeal swabs. Moreover, once a sample is obtained it can be grossly mistreated without influencing the positivity of the ELISA test. It was somewhat surprising to us, considering the reputation for heat lability that RSV carries, that as many as one-third of the secretions remained positive by culture after a week on a laboratory bench top and subjected to adverse conditions of light and temperature.

We hoped that ELISA, like IF (4), might show some advantages of sensitivity over culture late in infection. We were, however, disappointed in this. It appears that immunoglobulin A (IgA) and IgG, which we know appear as cultures become negative, serve to block the reaction or to clear from the secretion any antigen released late in the infection.
ACKNOWLEDGMENTS

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LITERATURE CITED