Detection of Respiratory Syncytial Virus in Nasopharyngeal Secretions by Inhibition of Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay developed for the demonstration of respiratory syncytial (RS) virus immunoglobulin G antibodies was used for the detection of RS virus in specimens of nasopharyngeal secretions (NPS) obtained from children with acute respiratory disease. Samples of NPS were incubated with a fixed amount of standard serum (human serum antibodies to RS virus) before being added to the enzyme-linked immunosorbent assay test plate. A decrease in the optical density value determined for this standard serum was seen with the majority of NPS specimens from which RS virus had been isolated in tissue culture. The reliability and the specificity of this inhibition test were supported by experiments with purified RS virus and by tests with NPS specimens containing other respiratory viruses.

The detection of respiratory viruses directly in nasopharyngeal secretions (NPS) from patients with acute respiratory disease is being studied by different techniques.

The successful demonstration of influenza, parainfluenza, and respiratory syncytial (RS) virus in NPS by immunofluorescent (IF) antibody methods has been achieved by several workers, as described in detail by Gardner and McQuillin (4).

The use of enzyme-linked immunosorbent assay (ELISA) for the detection of influenza and adenovirus in NPS is being explored under experimental conditions, and more sensitive assays are being developed (2, 7). Chao et al. (3) have detected RS virus by ELISA in NPS from infants with acute respiratory disease. These authors employed a sandwich technique with horse anti-RS serum as capture antibody and rabbit anti-RS serum as detector antibody (3).

Sarkkinen et al. (13) have recently developed a four-layer radioimmunoassay and a four-layer ELISA for the detection of RS virus, parainfluenza virus type 2, and adenovirus in NPS from children with acute respiratory disease.

A method for the detection of RS virus in NPS by inhibition of ELISA is described in the present report. An ELISA technique for the assay of RS virus immunoglobulin G (IgG) antibodies (8) could, with a few modifications, be employed for the demonstration of RS virus in NPS obtained from small children and infants with acute respiratory disease.

MATERIALS AND METHODS

Patients examined. We examined 84 infants and children admitted with acute respiratory disease to two pediatric departments in Copenhagen. The age of the patients varied from 1 month to 7 years and 7 months. Fifty-four of the patients were below 2 years of age. Eighty of the patients had lower respiratory disease (pneumonitis, bronchitis, or acute laryngitis), two had rhinopharyngitis, and two had influenza.

NPS examined. Samples of NPS were obtained within 24 h of admission by suction through a nasal catheter according to the methods described by Gardner and McQuillin (4). Suction was applied through each nostril, and finally 2 ml of transport medium (Parker medium 199, supplemented with 0.2% bovine serum albumin and adjusted to pH 7.0 with sodium bicarbonate) was sucked through the catheter. NPS samples were transported in a cooling container to the diagnostic laboratory within 3 h after sampling. All samples of NPS had been stored at 70°C for periods ranging from 4 to 16 months before being tested by ELISA.

Tissue culture. HEp-2 cells were grown and maintained in Roux bottles, in 12-by-100-mm plastic tubes, or in Layton tubes as described earlier (8). Primary monkey kidney cells (Cercopithecus) were grown and maintained in 12-by-100-mm glass tubes or Layton tubes as described earlier (9).

Examination of NPS for the presence of influ-
enza, parainfluenza, or RS virus by IF antibody technique. Fresh, nonfrozen NPS specimens were examined by the indirect IF technique according to methods described earlier (4, 6). The following virus-specific hyperimmune sera were employed: bovine anti-influenza A serum (Wellcome Research Laboratories, Beckenham, England), bovine anti-RS serum (Wellcome), and rabbit anti-parainfluenza virus type 1 serum (National Bacteriological Laboratories, Stockholm, Sweden).

Isolation of RS virus, influenza virus type B, and adenovirus. Within 4 h after sampling, NPS specimens were inoculated into HEp-2 and primary MK tissue cultures. RS virus was isolated in HEp-2 tube cultures. This virus could usually be identified by the typical syncytial cytopathic effect. Questionable cytopathic effect resulted in passage of medium to HEp-2 cultures in Layton tubes, where RS virus was identified by the IF technique (8). Adenovirus was also isolated in HEp-2 tube cultures. Cells showing possible adenovirus-like cytopathic effect were solubilized in tris(hydroxymethyl)aminomethane-barbital buffer (pH 8.6), ionic strength 0.05, containing 1% Triton X-100, and tested as antigen in countercurrent immunoelectroosmosis, in which rabbit antisera to group antigen was employed as antibody (6) by the technique described earlier (5). Influenza virus type B was isolated in primary MK cultures. Cultures showing positive hemadsorption after the addition of guinea pig erythrocytes were passed to new MK cultures. Medium from these secondary cultures was tested by hemagglutination inhibition by use of ferret antiserum raised against influenza A/Hong Kong/5/72.

Detection of RS virus antigen in samples of NPS by inhibition of ELISA. The technique employed (outlined in Fig. 1) has been described in detail earlier (8), where it was used for the assay of RS virus IgG antibodies. In the present investigation it was used for the detection of RS virus antigen, with a few modifications involving only the third layer (human serum layer) of this ELISA technique.

Dilutions of NPS were mixed with a human serum containing RS virus antibodies and incubated overnight (4°C). This mixture was subsequently added to the ELISA plate as the third layer of the test (Fig. 1). The decrease in the optical density (OD) values of the standard serum observed in some of the NPS specimens was interpreted as indicating the presence of RS virus antigen.

The standard serum used was the immunoglobulin fraction of a pool of 10 human sera showing medium to high RS virus complement fixation titers (8). This immunoglobulin fraction was adjusted to an immunoglobulin concentration of approximately 10 g/liter of normal saline. It had an RS virus neutralizing titer of 1:3,200 and an RS virus IF antibody titer of 1:160 (8).

For the test proper, equal volumes of samples of NPS diluted twofold (1:2 to 1:8) in ELISA dilution buffer (8) were mixed with standard serum diluted 1:1,000 and 1:2,000 and also in dilution buffer. These eight mixtures of NPS and standard serum were then added to the plate (0.1 ml per well) the following day (Fig. 1A) together with mixtures of standard serum (diluted 1:1,000, 1:2,000, 1:4,000, 1:8,000) and dilution buffer used as positive controls (Fig. 1B). The samples of NPS were briefly sonicated (10 s, 16 kilocycles per s, Branson sonifier) before being mixed with standard serum. The daily testing of the same dilutions of the same standard serum allowed for corrections of the day-to-day variations of the OD values obtained.

Other reagents employed in this ELISA technique were capture antibodies, RS virus antigen, and peroxidase-conjugated rabbit anti-human IgG, which have all been described in detail earlier (8).

Determination of RS virus IgG antibodies in samples of NPS. This was done by the ELISA technique described earlier (8) for serum IgG antibodies by substituting dilutions (1:4, 1:8, 1:16, and 1:32) of NPS for the human serum dilutions.

Purification of RS virus. RS virus was purified with maintenance medium from RS virus-infected tissue cultures (Roux bottles) as the starting material, according to the method described by Senterfit and Baldridge (14).

Electron microscopy. Estimation of the number of RS virions present in suspensions of purified virus was made according to the methods described (8, 12). Experience shows that definite numbers of virions are difficult to determine by electron microscopy of pleomorphic virus like RS virus. Since only easily recognizable virions of intact morphology were counted, the number of RS virions determined by electron microscopy in the present report represented a rough estimate of the minimum number present.

Growth of RS virus. The Long strain cultivated in Roux bottles with HEp-2 cells as earlier described (8) was employed for the following purposes: (i) for the production of cellular antigen used as the second layer in the ELISA test plates; (ii) for the production of purified RS virus; and (iii) for the production of RS virus-infected cells used for specificity tests.

HEp-2 cell suspensions for specificity tests. RS
virus-infected HEp-2 cells were obtained from Roux bottles by treatment of monolayers showing advanced cytopathic effect with ethylenediaminetetraacetate. Twofold dilutions of a cell suspension containing approximately 10⁶ cells per ml were made in ELISA dilution buffer (8). These cell suspensions were briefly sonicated (10 s, 18 kilocycles per s).

Standard serum used for specificity tests. The human standard serum referred to above was absorbed with uninfected HEp-2 cells. Five milliliters of standard serum, diluted 1:10 in ELISA dilution buffer, was absorbed overnight (4°C) with 10⁸ HEp-2 cells.

RESULTS

Pilot experiments with RS virus preparations. Experience concerning the reliability of the ELISA inhibition (EL-IN) test was gained from experiments performed with RS virus-infected HEp-2 cells and purified RS virus.

RS virus-infected HEp-2 cells. Equal volumes of RS virus-infected (sonicated) cells and standard serum (dilution, 1:1,000) were mixed and incubated overnight (4°C) before being added (0.1 ml of mixture per well) as a third layer to the ELISA test plate for 1 h. Mixtures of equal volumes of standard serum (dilution, 1:1,000) and uninfected (sonicated) HEp-2 cells were added to similar wells in the same plate, and mixtures of equal volumes of standard serum (dilution, 1:1,000) and dilution buffer were also added to the plate.

The results are seen in Fig. 2. When a suspension of sonicated RS-infected cells corresponding to approximately 2.5 × 10³ cells per ml was mixed with standard serum (diluted 1:1,000), inhibition of the OD value was seen, increasing to almost maximum inhibition when a concentration of sonicated RS-infected cells corresponding to approximately 25 × 10³ per ml was mixed with this dilution of standard serum. When different numbers of noninfected and sonicated HEp-2 cells were added to the standard serum, no inhibition of the OD values for the standard serum was observed. All OD values fell inside the area marked 2.

The concentration of RS virions in a suspension purified as described above was estimated by electron microscopy to be approximately 30 × 10⁴ per ml. As mentioned above, this number represents the count of easily recognizable virions of intact morphology, and the total number of virions of this very pleomorphic virus is possibly higher. This suspension was diluted fivefold in ELISA dilution buffer. Equal volumes of these suspensions and standard serum (dilution, 1:1,000) were mixed and incubated overnight before being added (0.1 ml per well) to the test plate. The results can be seen in Fig. 3. When suspensions containing more than 10⁴ easily recognizable RS virions per ml were incubated with the standard serum, inhibition was observed.

Examination of NPS containing different viruses by the EL-IN technique. Figure 4 shows the results obtained when samples of NPS from 84 infants and children were tested. All samples had been tested for the presence of virus by tissue culture (TC) and by IF antibody stain-

![ELISA Inhibition](http://jcm.asm.org)

**Fig. 2. OD values obtained when a fixed amount of human RS virus antibodies (standard serum diluted 1:1,000) was mixed with increasing amounts of RS virus-infected (and sonicated) HEp-2 cells (3). The OD values obtained by mixing standard serum and uninfected (and sonicated) HEp-2 cells were found within the hatched area (2), and the OD values obtained by mixing standard serum and dilution buffer were found within the hatched area (1). All of the mixtures were incubated overnight and subsequently added as the third layer to the ELISA test plate.**
were referred to the same viral antigen outline by Yolken et al. (15). Even though the sensitivity of this inhibition method could be increased by separation of the antigen-antibody complex by precipitation with polyethylene glycol 9,000, it was found to be less sensitive for cytomegalovirus antigen detection than both the single-antibody sandwich method and the double-antibody method (15).

A method for detection of RS virus in nasal secretions has been described by Chao et al. (3), who used a double-antibody method. These authors reported that RS virus was detected by ELISA in 79% of specimens found positive for RS virus by TC and that the ELISA technique seemed slightly more sensitive than the IF technique for RS virus detection (3). In the present report, the EL-IN test employed detected RS virus in 25 (61%) of 41 specimens found positive by TC, whereas the IF technique gave positive results in 27 (65%) of the specimens. We chose
as the base control value the mean OD value of a number of values obtained with negative secretions. Chao et al. (3) chose the OD value obtained with one single negative secretion as the base control value. Differences in estimation of OD values obtained, or perhaps differences in the sensitivity of HEp-2 cells employed for RS virus isolation, might explain the lower sensitivity of the EL-IN test as compared with that of the ELISA double-antibody test employed by Chao et al. (3). The double-antibody test was not evaluated on any specimens of NPS investigated in the present study because of lack of test material.

The reliability of the test described in this report was supported by experiments in which positive human standard serum was incubated with either purified RS virus or sonicated RS virus-infected HEp-2 cells. A good correlation was found between increasing amounts of virus and decreasing ability of the standard serum to bind to the ELISA test plate. Further support for reliability and specificity comes from the testing of different groups of NPS samples. No inhibition of the positive standard serum was observed by testing 23 NPS samples that were negative by TC and IF techniques or by testing 20 NPS samples containing other viruses.

According to McIntosh et al. (11), cell-free RS virus IgG antibody can usually be found in samples of NPS taken during the early phase of the disease, whereas cell-bound IgG antibodies are seldom present in early specimens. In the EL-IN technique presented in this paper, the detection of RS virus antigen rests on the inhibition of a fixed amount of human IgG antibodies incubated with the test specimen of NPS. It is theoretically possible that RS virus IgG antibodies present in NPS could interfere with the OD reading, rendering the inhibition caused by RS virus antigen less evident. Unfortunately not enough samples of NPS positive for RS virus by isolation in TC were available to study this problem further. In the group of NPS samples found positive for viruses other than RS virus, a tendency towards obtaining increased OD values was observed, as compared with the values observed in the group of NPS samples found negative for virus. Five of nine NPS samples (from the group of NPS samples containing other viruses) showed the presence of RS virus IgG antibodies. This might represent anamnes-

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**Fig. 4.** OD values obtained when specimens of secretions from four groups of children with acute respiratory disease were mixed with a fixed amount of human RS virus antibodies (human standard serum diluted 1:1000). The mean OD value for 23 secretions that were found negative for viruses by other techniques is indicated by \( \bar{x} \). This value minus 3 standard deviations is shown as \( \bar{x} - 3\sigma \). The number of children in the four groups is given in parentheses.
tic antibody responses provoked by other virus infections present in these five 1- to 7-year-old patients. According to McIntosh et al. (11), IgA antibody is not present in NPS during the early phase of illness in infants with RS virus infections. IgA antibody therefore should not be able to interfere with the EL-IN technique employed in the investigation reported here.

The purpose of this study has been to show that it is possible to use a sensitive and specific ELISA, adjusted to the detection of a standard amount of RS virus IgG antibodies, for the demonstration of RS virus antigen in NPS. This ELISA for IgG antibodies, described earlier (8), detects antibodies directed towards a few RS virus polypeptides. The possibility exists that it is primarily some of these RS virus polypeptides which are detected by the inhibition test outlined in the present report.

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LITERATURE CITED


