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Detection of an Immunoglobulin M Response in the Elderly for Early Diagnosis of Respiratory Syncytial Virus Infection

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The indirect fluorescent-antibody technique was compared with indirect and μ-capture enzyme-linked immunosorbent assays for the detection of respiratory syncytial virus (RSV) immunoglobulin M (IgM) in the elderly. Sera from 47 patients (mean age, 70 years) with acute lower respiratory tract infections caused by RSV were investigated. Specific IgM was detected in 81% (38 of 47) of the patients. The fluorescent-antibody technique, which gave 70% positive results, proved to be the most sensitive of the three methods. An IgM response was seldom seen in sera from the elderly within the first week of disease, but was present in 85% of sera (33 of 39) collected between days 11 and 30 of disease. In some patients it persisted for more than 6 weeks. Detection of IgM was found to be a useful tool for the diagnosis of RSV infections in elderly patients.

Respiratory syncytial virus (RSV) is known to cause severe respiratory tract infections in infants and small children. RSV has occasionally been reported to cause respiratory distress, as well as bronchitis and pneumonia in the elderly as well (3, 16, 18).

In children, viral antigen detection in nasopharyngeal secretions by the fluorescent-antibody (FA) technique is commonly used for the early diagnosis of RSV infections (5, 11). In the elderly, the respiratory mucosa is often dry and the mucus is viscous. This implies a decreased quality of the nasopharyngeal secretion preparation and the necessity of serological confirmation of the diagnosis by the complement fixation (CF) test or by enzyme-linked immunosorbent assay (ELISA). A specific immunoglobulin M (IgM) response to RSV in children is reported to develop early in the disease (1, 19). So far, no information is available on the appearance of specific IgM in RSV infections among the elderly.

The aim of the present study was to investigate the IgM response to RSV infections in the elderly. Three different methods were evaluated: the FA technique, indirect ELISA, and μ-capture ELISA.

MATERIALS AND METHODS

Patients. Adult patients with suspected pneumonia admitted to the Department of Infectious Diseases, Örebro Medical Center Hospital, Örebro, Sweden, were tested routinely for RSV antibody titer rises by the CF test. During the period from 1971 to 1980, 57 patients were diagnosed with a RSV infection by a fourfold CF titer rise between acute- and convalescent-phase sera or by a single high CF titer of ≥80. Sera from 47 of the patients were available for analysis of the presence of RSV-specific IgM. All sera in the study group were tested by the CF test for the following etiologies: influenza A virus, influenza B virus, adenovirus, *Mycoplasma pneumoniae*, and *Chlamydia* species. Results of these analyses were negative for all patients. The mean age of the patients was 70 years. Fourteen patients were more than 80 years of age. Pneumonia was diagnosed in 33 of 47 patients. The diagnosis was based on clinical signs and confirmed by pulmonary infiltrates on roentgenograms. The remaining 14 patients had symptoms of severe lower respiratory tract infection but a negative chest X ray and were regarded to have severe bronchitis.

Controls. Controls consisted of 35 sera from 18 patients with infections caused by any of the following agents: influenza A virus, influenza B virus, parainfluenzae virus type 3, mumpsvirus, adenovirus, and *M. pneumoniae*. The diagnoses were determined by the CF test. All sera were negative for RSV by the CF test.

Detection of specific IgM. Three different methods were used for the detection of RSV-specific IgM. Before sera were tested by immunofluorescence or indirect ELISA, they were absorbed with protein A-Sepharose (Pharmacia, Uppsala, Sweden) as described previously (15).

(i) FA technique. Monolayers of Vero cells grown on cover slips (4 cm^2) were inoculated with RSV (Long strain). The virus dose was selected to give 50 to 100 foci each 5 to 10 infected cells on the cover slips in 48 h. After fixation in anhydrous acetone for 5 min at room temperature, the cover slip with its infected tissue culture was stored in sealed plastic tubes at −70°C until use.

Sera treated with protein A-Sepharose were applied in twofold dilutions ranging from 1:8 to 1:128 to the fixed RSV-infected tissue culture. After incubation for 90 min at 37°C and three washings (10 min each) in 0.01 M phosphate-buffered saline (PBS; pH 7.2), fluoroisothiocyanate-conjugated rabbit anti-human IgM serum at a dilution of 1:15 (Dakopatts, Copenhagen, Denmark) was added and incubated at room temperature for 20 min. After repeated washings, the cell preparations were counterstained for 4 min in Evans blue (1:30,000). Examination was performed at ×400 magnification in a fluorescence microscope (Zeiss) with incident light. Only sera giving the typical granular fluorescence of the cytoplasm of the infected cells were regarded as positive.

(ii) Indirect ELISA. RSV (Long strain) was grown in Vero cells, and the supernatant was collected for subsequent concentration of virions by ultracentrifugation (14).

The wells of a microdilution plate (M129A; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 0.1 ml of RSV antigen (protein content, 40 μg/ml). Sera for testing (treated with protein A-Sepharose; Pharmacia) were diluted 10-fold from 1:50 to 1:5,000 in 0.01 M PBS (pH 7.2) containing 0.05% Tween 20 (PBS-Tween) supplemented with 0.5%

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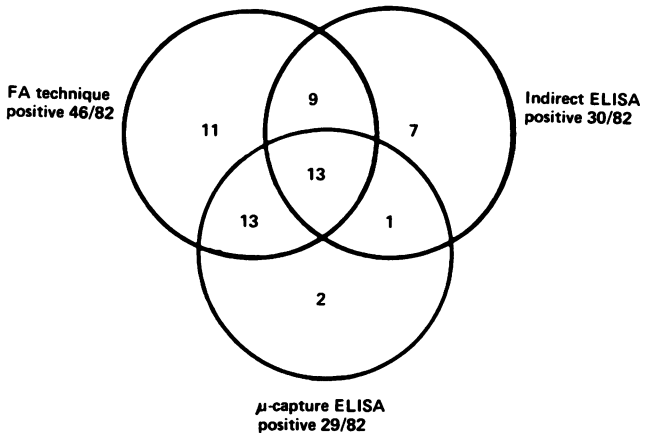

FIG. 1. IgM detection in 82 sera from 47 patients by the FA technique, μ-capture ELISA, and indirect ELISA. The numbers inside the circles represent sera that were IgM positive in one, two, or three of the assays.

bovine serum albumin and applied in 0.1-ml portions to the coated wells. The plate was incubated for 3 h at 37°C. After the wells were washed in PBS-Tween, alkaline phosphatase-conjugated swine anti-human IgM serum (Orion, Espoo, Finland) was added to the wells, and the plate was again incubated for 3 h at 37°C. Repeated washings in PBS-Tween were followed by the addition of 0.1 ml of the substrate *para*-nitrophenylphosphate (0.5 mg/ml; 104; Sigma Chemical Co., St. Louis, Mo.). The plate was incubated for 30 min at room temperature before the reaction was stopped by the addition of 0.025 ml of NaOH (3 M). The A_{405} values were read in a spectrophotometer (Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.). The cutoff value for positivity was calculated as the mean of 20 negative sera plus 2 standard deviations.

(iii) μ-capture ELISA. RSV (Long strain) was conjugated with horseradish peroxidase as described by Wilson and Nakane (20).

For the assay the wells of a microdilution plate (M29AR; Dynatech) were coated with 0.1 ml of rabbit anti-human IgM serum (μ-chain specific; Dakopatts, Copenhagen, Denmark) diluted 1/500 in carbonate-bicarbonate buffer (pH 9.6) and incubated for 3 h at 37°C. After sera were washed three times with PBS-Tween, sera for the test were diluted 10-fold from 1:50 to 1:5,000 in PBS-Tween-bovine serum albumin and added to the wells in 0.1-ml portions. After incubation for 2 h at 37°C, the plate was again washed three times with PBS-Tween, and the peroxidase-labeled RSV antigen was added. The plates were incubated further for 2 h at 37°C and subsequently washed. The substrate 5-aminosalicylic acid was added, and the color reaction was read after 30 min at 450 nm in a spectrophotometer (Titertek Multiskan; Flow Laboratories). The results were expressed as positive or negative with reference to the calculated cutoff level, determined as twice the background activity.

Rheumatoid factor. All sera were tested for the presence of rheumatoid factor (Cooper Biomedical Inc.).

RESULTS

RSV IgM was detected by the FA technique in 46 of 82 sera, which represented 33 of 47 (70%) patients. The corresponding results for indirect ELISA were 30 of 82 sera, i.e., 24 of 47 patients (51%), and for the μ-capture ELISA, 29 of 82 sera, i.e., 22 of 47 patients (47%). IgM was detected by any of the three methods in 56 of 82 sera, which represented 38 of 47 (81%) patients. The results obtained by the three methods for all sera are given in Fig. 1.

TABLE 1. Results of three assays for RSV IgM in sera taken on admission[a]

Days after onset of RSV disease	Total no. of serum samples	No. of sera positive by:			
		FA technique	Indirect ELISA	μ-Capture ELISA	CF ≥ 80
≤5	15	0	1	0	0
6–10	12	9	6	4	6
11–15	7	5	1	2	5[b]
16–20	5	5	4	4	5
>20	7	4	2	3	4[c]
Total	46[d]	23	14	13	20

[a] The FA technique, indirect ELISA, and μ-capture ELISA were compared with the CF test at different times (in days) after the onset of disease caused by RSV.
[b] One serum sample was negative in the IgM test.
[c] Three serum samples were negative in the IgM test.
[d] Acute-phase serum from one patient is missing.

Results of the CF test for sera taken on admission to the hospital compared with the results of the three different IgM assays are given in Table 1.

The sensitivity of each of the three methods, calculated by using sera that were positive by the other two methods as an arbitrary positive reference for the presence of RSV IgM, was 97% for the FA technique, 75% for μ-capture ELISA, and 64% for indirect ELISA (Table 2). The specificities, calculated by using sera that were negative by the other two methods as a negative reference, are given in Table 2. RSV IgM was not found by any of the three methods in 35 sera from patients with other serologically verified viral infections or mycoplasmal disease.

The IgM response to RSV was seldom detected before day 6 of illness. During the following 4 weeks, IgM was present in about 75% of the sera. The results of the three IgM tests for 74 of 82 sera sampled at different times up to 40 days after the onset of symptoms are presented in Fig. 2. Of the eight sera sampled more than 40 days after the onset of symptoms of RSV infection, IgM was detected in four.

TABLE 2. Sensitivity and specificity of the FA technique, indirect ELISA, and μ-capture ELISA for RSV IgM[a]

Test	No. of sera with results in ≥2 tests			Sensitivity	Specificity
	Positive	Negative	Total		
FA technique					
Positive	35	11	46	35/36 = 0.97	35/46 = 0.76
Negative	1	35	36		
Total	36	46			
Indirect ELISA					
Positive	23	7	30	23/36 = 0.64	39/46 = 0.85
Negative	13	39	52		
Total	36	46			
μ-Capture ELISA					
Positive	27	2	29	27/36 = 0.75	44/46 = 0.96
Negative	9	44	53		
Total	36	46			

[a] Sera that were positive or negative by two of the methods were used as arbitrary references.

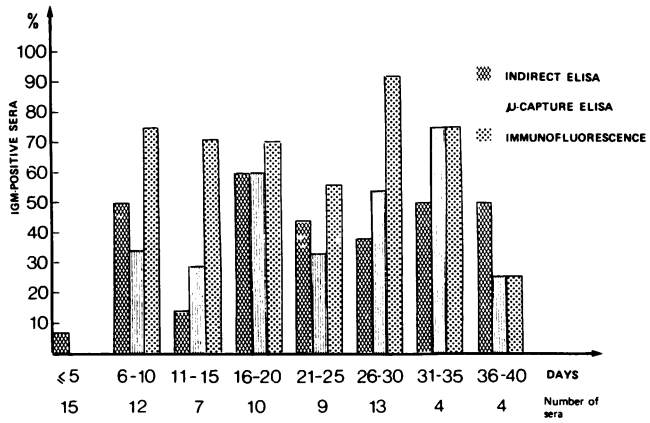

FIG. 2. Frequency of RSV IgM-positive sera sampled at different time intervals from the onset of symptoms and analyzed by the FA technique, μ-capture ELISA, and indirect ELISA.

Convalescent-phase sera (mean sampled 28 days after the onset of symptoms; range, 15 to 39 days) from six elderly patients (mean age, 74 years; range, 64 to 97 years), who were chosen randomly among patients fulfilling the criteria of a fourfold rise of the CF test titer against RSV, were compared with sera (mean sampled 32 days after the onset of symptoms; range, 16 to 57 days) from children with a RSV infection diagnosed by a fourfold increase of the CF test titer or by antigen detection in nasopharyngeal secretions. The children (mean age, 8 months; range, 5 to 10 months) were chosen to match the elderly patients in days after the onset of symptoms to the sampling day. The IgM response in elderly patients and children was compared by studying the absorbances by indirect ELISA and μ-capture ELISA at a serum dilution of 10^{-3}. No statistically significant differences were demonstrated by the Wilcoxon rank sum test. Consistent results were obtained by the FA technique.

Patients with RSV infections confirmed by a significant antibody titer rise (determined by the CF test) but without detectable IgM were studied separately. No difference in age distribution, occurrence of pneumonia, degree of illness, or duration of disease could be seen in these patients compared with the IgM-positive patients.

Rheumatoid factor was not detected in any of the sera.

DISCUSSION

Early diagnosis of many viral infections can be achieved by the detection of specific IgM in a single serum specimen. However, there are difficulties associated with the detection of virus-specific IgM. In indirect tests IgG may inhibit the binding of IgM to the antigen in the solid phase, giving false-negative results. Furthermore, the rheumatoid factor of the IgM class may cause false-positive results by reacting with antigen-IgG complexes. These difficulties can be circumvented by removal of IgG and rheumatoid factor from the serum or by separating the IgM from other immunoglobulins in the serum for testing.

The μ-capture ELISA has previously been found to be useful for the detection of specific IgM in various viral infections (2, 4, 10, 17). The principle of this method involves the separation of IgM from serum on a solid phase, followed by detection of specific IgM by labeled antigen. The influence of the rheumatoid factor can thus be avoided.

The frequency of a positive IgM response among the elderly to RSV infection is not known. Because there is no standard reference test for RSV IgM, the sensitivity of the different tests could be calculated only if the sera which were positive by two of the methods were assumed to be true IgM positives. The sensitivity obtained by the FA technique was 97%, for μ-capture ELISA it was 75%, and for the indirect ELISA it was 63%. The FA technique was also shown to be the most rapid of the three methods. The calculated specificity for the FA technique was only 76%; however, if a reference test with a lower sensitivity compared with that of the evaluated method was used, a false low specificity of the method being studied would result. Based on our results and those of other studies (8, 19), the FA technique is highly specific. An additional confirmation of the specificity of the FA reaction was obtained by the observation of the appearance and distribution of the fluorescence in the infected cells. This was judged to be the main reason why low titers of antibodies could also be reliably diagnosed.

The IgM response to primary RSV infections in children has previously been studied by the FA technique. Cranage and Gardner (1) detected IgM in 8 of 13 (62%) cases, and Welliver et al. (19) detected IgM in 5 of 22 (23%) cases during the first 4 days of illness and in 13 of 18 (72%) cases at days 5 to 9. After 1 year, no IgM could be detected. Reinfection with RSV at an interval of 1 year was shown to result in an accelerated IgM response (19). However, Meurman et al. (12) reported a weak or absent IgM response in reinfections in children. No explanation was given for these contradictory findings. In a prospective study of RSV infections in children, Hendersen et al. (7) found a high attack rate (98%) for primary RSV infections during RSV epidemics. The majority of our patients probably suffered primarily from RSV infections and reinfections during childhood. The time span to the investigated RSV infection in adulthood was probably long enough to provoke an antibody response with a primary response character.

Hornsleth and co-workers (8) have previously compared the FA technique and μ-capture ELISA for the detection of specific IgM in serum in children with primary RSV infections. By the FA technique IgM was detected in 18 of 22 (82%) children and by ELISA in 20 of 22 (91%) children. The FA technique was found to detect IgM for a longer period after the onset of symptoms as compared with the ELISA. In the present study, specific IgM in the elderly with a RSV reinfection was detected in 33 of 47 (70%) patients by the FA technique and in 22 of 47 (47%) patients by the μ-capture ELISA. An IgM response before day 5 was seen in only one case. Of 12 sera, 9 (75%) were IgM positive on days 6 to 10 and 29 of 39 (75%) sera were IgM positive on days 11 to 30 by the FA technique. The time period between the onset of symptoms and the detection of RSV IgM did not significantly differ from that which has been described for children (19). Results of the present study offer no facts that explain the lack of an IgM response, in any of the assays, in about 20% of the elderly infected with RSV. Nor did we find any differences in the strength of the IgM response measured by any of the three methods in the elderly compared with that in the children.

From results of a study on primary RSV infections in children, Hornsleth et al. (8) concluded that antigen detection is more sensitive than antibody detection. However, Meurman and co-workers (13) have reported serology to be more sensitive than antigen detection. Hall et al. (6) have reported that the virus shedding phase is shorter in adults than in children (1.6 versus 3.9 days, respectively). The respiratory mucosa is often dry in older patients, even when it is infected, and the mucus is more viscous in the elderly

than in children (9). These factors, combined with a later admission of the elderly with RSV infections to the hospital, have made it impossible to obtain a study group from which RSV can be isolated and used as a reference for serological studies. In conclusion, a serum antibody analysis may, at this point, be the best alternative for the early diagnosis of RSV. The results of the present study show that detectable RSV IgM in adults seldom appears in serum before day 5 of the disease, and generally not before day 7. This may seem to be a serious limitation of the usefulness of the IgM detection method as an early method for the diagnosis of RSV infections among the elderly. However, 31 of 47 (66%) patients in this study were admitted to the hospital more than 5 days after the onset of symptoms. Five days after the onset of symptoms may be too late to reliably detect viral antigens from the nasopharyngeal secretions, and the IgG titer rise may not yet be diagnostic. The results of the present study indicate that in about 70% of the patients, the diagnosis was confirmed about 1 week after the onset of symptoms by detection of specific IgM in a single serum sample.

The early correct diagnosis of RSV disease is important for epidemiological reasons and to prevent unnecessary antibiotic treatment in cases in which complicating bacterial infections are unlikely. Furthermore, an early diagnosis may give support for specific antiviral treatment in the future.

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