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DETECTION OF RESPIRATORY SYNCYTIAL VIRUS SERUM ANTIBODIES BY AN ELISA SYSTEM

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Summary. — An ELISA test for respiratory syncytial (RS) virus assay was adapted and standardized; it gave 10—15 times higher antibody titres than complement fixation (CF) but was not a more sensitive test for detecting recent RS virus infection in persons above 1 year of age. In testing normal-population sera, ELISA revealed twice more positive sera than the CF test. Owing to its high sensitivity and apparent ability to detect long-persisting antibodies, ELISA is the test of choice for sero-epidemiological surveys on RS virus infections.

Key words: respiratory syncytial virus; *enzyme immunoassays*; *ELISA*; *seroepidemiological surveys*

Introduction

The study of numerous as yet unresolved problems in the area of immunology and pathogenesis of respiratory syncytial (RS) virus (genus *Pneumovirus*) infections, and the difficulty of their aetiological diagnosis, especially in the youngest children, call for the use of laboratory tests of the highest possible sensitivity (Chanock *et al.*, 1976). We thus have taken recourse to the ELISA technique, elaborating it into a highly sensitive system for measuring RS virus antigen-specific antibody reactions. The results obtained are presented below.

Materials and Methods

Human sera. The following sets of human sera were tested for RS virus antibodies: *a*) 15 pairs of acute and convalescent sera from children 1—10 years of age ill with acute respiratory disease (ARD) of RS virus aetiology as demonstrated by virus isolation or specific antibody rise in the complement-fixation (CF) test or both methods; *b*) 67 serum pairs collected from children 1—10 years of age with ARD of unidentified viral aetiology, and *c*) 208 sera obtained for immunological-survey purposes from different age groups of the normal population of one area in 1979.

ELISA. The assay was performed in flat-bottom microplates as a modification of the techn'que described by Voller *et al.* (1976). All reagents were added in 200-μl amounts per well. *a*) *Preparation of antigen.* The prototype RS virus strain Long was cultivated in L-132 cells in Eagle's minimum essential medium with 2% calf serum. After attaining a complete cytopathic effect, the cell cultures were frozen, thawed and centrifuged at 8,000 rev/min for 10 min. The supernatant was stored and the cell sediment sonicated twice for 2 min (at 10 KC). The sonicated material was spun to remove cell debris and the supernatant was joined with that of the first centrifugation

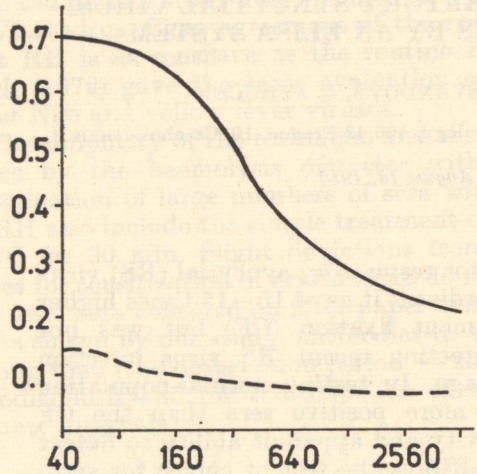

Fig. 1.

Standard curves of a positive (——) and a negative (— — —) serum
Abscissa: serum dilution reciprocals; ordinate: OD_{450} values

run. The resulting fluid was centrifuged at 120000 × g for 3 hr. The pellet of intra- and extracellular virus thus obtained was dissolved in phosphate buffered saline (PBS) pH 7.2, in 1/50 of the original volume. Optimum antigen dilution for the test itself was determined by titrations against a known positive and negative serum: that antigen dilution which on the titration curve gave the greatest difference between positive and negative samples was used as the working dilution in all subsequent tests. *b*) *Antigen adsorption* (sensitization of microplates) was performed at 4 °C overnight with the antigen diluted 1 : 50 in carbonate buffer pH 9.6 and with 0.02% NaN_3 added. *c*) *Washing procedures.* Between the individual test steps, all wells of each plate were carefully washed 3 times with PBS, pH 7.2, containing 0.05% Tween-20. *d*) *Sera tested.* Initial serum dilution was 1 : 40 in PBS, pH 7.2, plus Tween-20. Incubation in sensitized microplates proceeded at 4 °C overnight. *e*) *Anti-human immunoglobulin.* Horse radish peroxidase-labelled IgG (Miles Laboratories) diluted 1 : 1000 was used. The plates were incubated at room temperature for 3 hr. *f*) *Enzyme substrate.* The substrate was prepared by dissolving 180 mg of

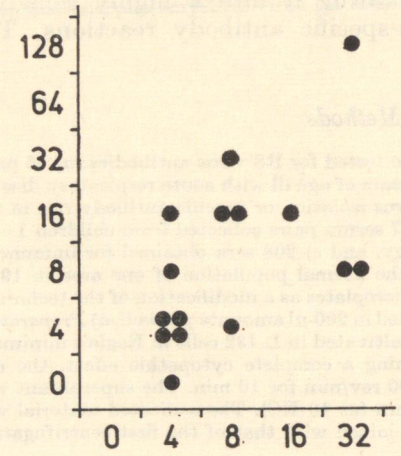

Fig. 2.

Rises of RS virus antibody titres in paired sera from children with ARD
Abscissa and ordinate: rises of antibody titres (-fold) determined by CF and ELISA, respectively.

5-aminosalicylic acid in 100 ml of distilled water heated to 70 °C, adjusting the pH to 6.0 with 1 N NaOH, and adding 20 µl of H_2O_2 immediately before instilling the substrate in the plates. By the action of peroxidase the substrate became dark brownish. *g*) The results were read in a photometer especially devised for measuring absorption of the contents of individual plate wells in situ (ELISA Reader, Dynatech) at 449 nm, or by naked eye. The test was standardized by repeatedly titrating a known positive and negative serum and comparing the results read visually and photometrically.

The complement fixation (CF) test was done on microplates with 4 antigen units as recommended by Chanock *et al.* (1961) for RS virus CF antibody detection.

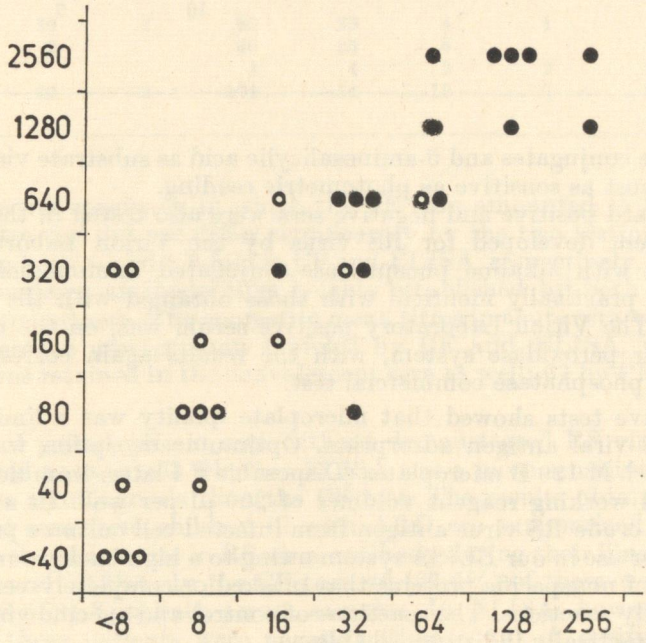

Fig. 3.

RS virus antibody titres in paired sera from children with ARD of RS virus aetiology
○ — Acute sera, ● — convalescent sera
Abscissa: CF titres, ordinate: ELISA titres

Results

Standardization of the ELISA system

Fig. 1 presents the titration curve for a known positive and a negative serum obtained by measuring optical density (OD) at 449 nm. In serial serum testing, the highest serum dilutions giving values of OD $\geqq$ 0.3 were considered positive. In visual estimation, using a four-cross colour scale, the OD 0.3 value was taken equivalent to two crosses (positive). With the use

Table 1. Correlation of CF and ELISA titres of RS virus antibodies in paired sera from children with ARD of unidentified viral setiology

No. of sera with CF titres of	No. of sera with ELISA titres of					
	<40	40	80	160	320	640
<8	19	4	15	6	1	1
8			3	1	1	
16				10	7	
32						
64						1

of peroxidase conjugates and 5-aminosalicylic acid as substrate visual estimation was almost as sensitive as photometric reading.

The standard positive and negative sera were also tested in the Dynatech ELISA system developed for RS virus by the Virion Laboratory. This assay works with alkaline phosphatase-conjugated immunoglobulins. The results were practically identical with those obtained with the peroxidase conjugates. The Virion Laboratory positive serum was, on the other hand, tested in our peroxidase system, with the results again corresponding to those of the phosphatase commercial test.

Comparative tests showed that microplate quality was a limiting factor for good RS viral antigen adsorption. Optimum adsorption was obtained with Dynatech M 129 B microplates (Disposable F Plates, Immulon Grade B) and minimal working reagent volumes of 200 μl per well. In a number of experiments crude RS virus antigen from infected cell cultures proved to be unsuitable for use in our ELISA system owing to a high background reaction, i.e. binding of nonspecific proteins that masked or completely veiled specific virus-antibody reactions. The reactions of control and specific viral antigens were often practically indistinguishable.

Sera in low dilutions sometimes reacted nonspecifically, but these reactions could be excluded by diluting the sera. In our tests we therefore used an initial serum dilution of 1 : 40.

Sensitivity of the ELISA system

To check the sensitivity of the assay system in terms of detection and titre of specific antibody, we tested:

a) A set of paired, acute-stage and convalescent sera from cases of RS virus aetiology to show to what degree the assay is capable of detecting recent RS virus infections. Specific RS virus antibody titre rises as revealed by the CF test and the ELISA system are compared in Fig. 2. In 14 out of 15 of the serum pairs tested, at least a fourfold rise in antibody titre was demonstrated by both tests. In one instance such a rise was only shown by the CF test; in this case a titre of 320 was recorded by ELISA already in

Table 2. RS virus antibody titres among the normal population as demonstrated by CF and ELISA

No of sera with CF titres of	No. of sera with ELISA titres of							
	<40	40	80	160	320	640	1280	Total
<8	30	2	63	25	4	1		125
8	2		40	25	6			73
16			1	4	2	2	1	10
Total	32	2	104	54	12	3	1	208

the acute serum specimen, in which the CF titre amounted to 8. The mean antibody titre rise did not differ significantly by the two testing procedures used, having been 8- and 8.8-fold in CF and ELISA, respectively.

Fig. 3 compares antibody titre heights established by both tests in the same set of paired sera. The geometric mean titres in acute sera were 13.7 and 160 (a difference greater than tenfold) by CF and ELISA, respectively. This ratio was retained in the convalescent sera as well: 67 by CF and 926 by ELISA.

b) A set of paired patient sera without demonstrated RS virus aetiology was examined to ascertain whether ELISA was a more sensitive test for RS virus disease serodiagnosis than the CF test. The results obtained on a set of 68 paired sera from children ill with ARD are summarized in Table 1. All patients showed no rise in RS virus antibody during their illness according to both CF and ELISA. In the CF test, 46 (67.6%) of them were negative, with a titre of < 8 in both serum samples. In ELISA, however, only 19 (27.9%) of these patients were negative (titre < 40). A positive antibody titre by CF was obtained in 22 children (geometric mean titre 14.6), by ELISA the number of positives was more than twice as high (49) and the geometric mean titre equalled 131.

c) A set of normal-population sera was tested to determine to what degree ELISA is suitable for detecting anamnestic titres of long-persiting IgG type specific antibodies. The results obtained on 208 such sera, collected from different age groups in one area in the Czech Socialist Republic, are presented in Table 2. In the CF test 83, i. e. 39.3%, of the sera were positive (titre ≥ 8) giving a geometric mean titre of 8.7. In ELISA, there were 176 (84.6%) positive sera (titre ≥ 40) and the geometric mean titre was 114.

Identical negative or positive results in the CF and ELISA test were obtained with 55.1% of 276 sera from both the normal population and children with ARD of undetermined virus aetiology. ELISA disclosed 43.5% more positive serum samples than did CF. Two sera (0.7%) were negative in ELISA but gave a titre of 8 by CF.

Discussion

Because of their sensitivity enzyme immunoassays, in particular ELISA, have recently become used for the detection of antibodies to a large number of viruses for purposes of both aetiological diagnosis and seroepidemiology, e. g., influenza A and B and parainfluenza 1 (Bishai and Galli, 1978; Oshin, 1979); herpes simplex virus (Gilman and Docherty, 1977; Vestergaard and Grauballe, 1979; Denoyel *et al.*, 1980); cytomegalovirus (Schmitz *et al.*, 1977; Sarov *et al.*, 1980); varicella-zoster virus (Goldberg, 1980); rotavirus (Ghose *et al.*, 1978). Although the general principles of the assay remain invariable, its successful application requires the elaboration and observance of specific conditions for each individual virus-antibody system.

So far, ELISA has been used for measuring the antibody response in RS virus infection only by Richardson *et al.* (1978), who tested a group of 91 children suffering from disease of the lower respiratory tract of RS virus aetiology. They used alkaline phosphatase-conjugated immunoglobulins and found that ELISA gave 100-fold higher titres than CF and 2—4-fold higher values than the plaque-reduction test. In our own material, ELISA titres were 10—15-fold higher than CF titres, similar to the results reported by Denoyel *et al.* (1980) concerning herpes simplex virus and by Bishai and Galli (1978) concerning influenza A and B and parainfluenza viruses. The above difference in titre height may be attributed to Richardson *et al.* (1978), having worked with crude antigen from infected cell cultures, which produced high-level nonspecific reactions (as compared with control antigen). These authors therefore started antibody titration with an initial serum dilution of 1 : 100, but some sera nevertheless still reacted nonspecifically. In our opinion, the use of crude virus antigens is not suitable for enzyme immunoassays.

It seems that the selection of the enzyme conjugate-enzyme substrate system does not influence the sensitivity of the test in a decisive way. We verified this by cross-testing positive and negative reference sera in our own peroxidase system and the Dynatech system with alkaline phosphatase-conjugated immunoglobulins. The practically identical results support the above conclusion, but its full confirmation would require verification on material of a larger size.

The finding by Richardson *et al.* (1978) that ELISA is more sensitive than the CF and plaque-reduction tests for immune-response detection in the youngest infants up to 6 months of age could not be confirmed in the present study because we possessed no sera from such young subjects. Our children tested were more than 1 year old and, in agreement with the authors cited, we found in them no difference between antibody titre rises detected by the CF test and ELISA.

Our findings in conjunction with those of Richardson *et al.* (1978) thus justify the conclusion that ELISA is the test of choice for detecting recent RS virus infections in infants up to 6 months old, but in older children and adults the CF test and ELISA are of equal value, even if ELISA gives substantially higher antibody titres.

But the situation becomes quite different in seroepidemiological investigations, in which the CF test detects antibodies persisting for a short time after infection only, while ELISA is capable of monitoring long-persisting antibodies, especially of the IgG type. In our testing of normal-population sera, ELISA revealed double that number of positive sera detected by the CF test.

The high rates of RS virus antibody prevalence found are in accord with the generally known fact that practically the entire child population is exposed to RS virus infection already at a very young age. Apparently, the individual retains lifelong traces of this early infection, even if in the form of a low antibody titre. There is good evidence, though, that both children and adults frequently undergo reinfection which for the most part takes a milder clinical course than the primary infection or is outright inapparent.

Thus, ELISA is again the test of choice for demonstrating circulation of RS virus among the population, especially in areas in which basic data on its prevalence and distribution are missing.

It may be concluded, therefore, that ELISA is a sensitive test for detecting RS virus antibodies, with a broad spectrum of applications, but one must be aware of all its pitfalls and possibilities, and its use may be recommended especially where the more usual methods cannot provide the results desired or answers to the points in question.

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