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Formalin-Inactivated Respiratory Syncytial Virus Vaccine Induces Antibodies to the Fusion Glycoprotein That Are Deficient in Fusion-Inhibiting Activity

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The fusion (F) glycoprotein of respiratory syncytial virus (RSV) induces neutralizing antibodies and antibodies that inhibit fusion of infected cells (FI antibody). It was previously shown that infants and children immunized with Formalin-inactivated RSV 20 years ago developed antibodies that bound to the F glycoprotein but were deficient in neutralizing activity. A reexamination of these sera indicated that they were also deficient in FI activity. Thus, Formalin-inactivated RSV vaccine stimulated an unbalanced immune response in which an unusually large proportion of the induced antibodies were directed against nonprotective epitopes rather than against the epitopes that induce functional antibodies, i.e., neutralizing and FI antibodies. This deficiency in stimulation of functional antibodies probably decreased the protective efficacy of the vaccine and could have contributed to potentiation of disease in the vaccinees during subsequent RSV infection.

Respiratory syncytial virus (RSV), which is in the pneumovirus subgroup of the *Paramyxoviridae* family (8), is the leading cause of severe viral lower respiratory tract disease in infancy (3, 5). Thus far, attempts to develop a live or inactivated vaccine effective against RSV have been unsuccessful (1, 2, 4, 14). Approximately 20 years ago, a Formalin-inactivated RSV vaccine was evaluated in infants and young children (1, 3, 4). This vaccine stimulated moderately high levels of serum antibodies, as measured by the complement fixation assay, but failed to induce resistance to infection or disease caused by RSV (1, 3, 4). In fact, vaccinees who received Formalin-inactivated RSV during early infancy developed more serious lower respiratory tract disease when infected with the virus than did individuals who received Formalin-inactivated parainfluenza virus vaccine (4).

It was shown recently that cotton rats inoculated intramuscularly with Formalin-inactivated RSV vaccine developed an increase in pulmonary pathology when subsequently infected by the intranasal route with RSV (9). In contrast, control unvaccinated animals or animals previously infected intranasally with RSV failed to develop significant pulmonary pathology following challenge. The animals that received the Formalin-inactivated vaccine developed pulmonary lesions, although virus replication in the respiratory tract was reduced approximately 95% compared with control animals. An examination of the immunogenicity of the Formalin-inactivated RSV vaccine in cotton rats revealed that these animals developed levels of antibodies to the large (G) and fusion (F) glycoproteins of RSV comparable to those attained in animals infected with RSV (9). However, the vaccinated cotton rats developed a level of neutralizing antibodies that was only 1/30th that of animals infected with RSV. Because the G and F glycoproteins are the major antigens to which neutralizing antibodies are directed (8, 10, 13), it appeared that most of the antibodies against these glycoproteins produced by the vaccinated animals lacked functional, i.e., neutralizing, activity. The sera of infants and children inoculated 20 years ago with Formalin-inactivated RSV vaccine were examined and found to contain moderate titers of enzyme-linked immunosorbent assay (ELISA)-binding antibodies that were deficient, like the cotton rat sera, in neutralizing activity (7). Thus, Formalin treatment of RSV appears preferentially to alter the F and G glycoprotein epitopes that stimulate neutralizing antibodies.

The F glycoprotein stimulates two types of functional antibodies: neutralizing antibody and syncytia- or fusion-inhibiting (FI) antibody (11, 12). Sera from cotton rats immunized with Formalin-inactivated vaccine developed ELISA-F antibody that lacked FI activity (9). We sought to extend these previous observations in animals by using a new FI assay that is highly specific for FI activity by examining sera obtained 20 years ago from the infants and children immunized with Formalin-inactivated RSV vaccine. Their FI and ELISA antibody responses were compared with those of infants and children naturally infected with RSV. The findings from the present study indicate that the RSV vaccinees developed ELISA-F antibodies that were deficient in FI activity.

Twenty-one infants between 2 and 7 months of age who attended a Child Health Center at the Children's Hospital National Medical Center were administered three doses of Formalin-inactivated RSV vaccine as described previously (4). Each vaccinee received two 0.5-ml intramuscular injections of RSV vaccine 1 month apart, and a third injection was given 3 months later. Serum was collected prior to immunization and 1 month following each immunization. The average age at the time of collection of the last serum sampling was 8.7 months. The responses of the RSV vaccinees were compared with those of a group of 15 infants and children aged 4 to 21 months (mean, 11.6 months) who were naturally infected with RSV. Eleven of these 15 naturally infected infants or children were hospitalized at Children's Hospital National Medical Center with RSV bronchiolitis or pneumonia in the late 1960s, and 4 were control Formalin-inactivated parainfluenza virus vaccinees who became infected with RSV in the epidemic of 1966 to 1967 (4).

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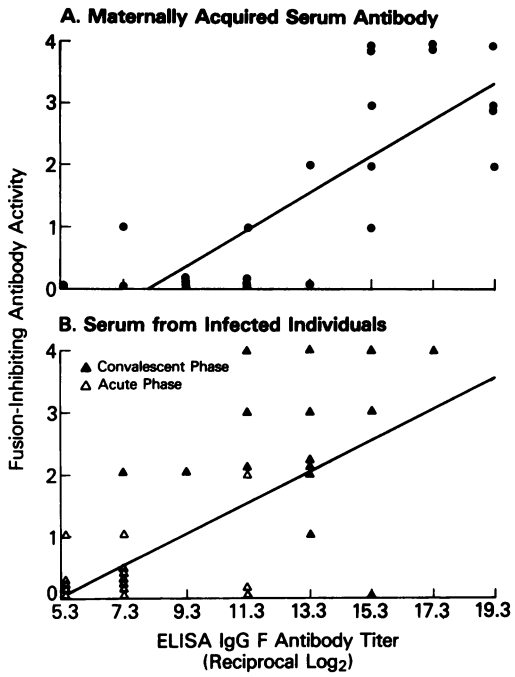

FIG. 1. Correlation between the ELISA IgG F antibody titer and FI antibody titer. (A) Serum samples were obtained before immunization from RSV vaccinees aged 2 to 7 months that contained maternally derived antibodies. The regression line indicates a positive correlation ($P < 0.001$). (B) Sera from infants and children who were not immunized with Formalin-inactivated vaccine but who were naturally infected with RSV were studied. The regression line indicates that there was a positive correlation ($P < 0.001$). The slopes of the regression lines in panels A and B are similar, 0.30 and 0.28, respectively.

The ELISA to detect F antibody in the immunoglobulin G (IgG) isotype was carried out as described previously (6). The fusion inhibition assay was performed with 10^5 HEp-2 cells, which were set onto glass cover slips in 24-well Costar plates. After 48 h, when the cells were approximately 75% confluent, they were infected with the Long strain of RSV at a multiplicity of infection (MOI) of 0.001 and supplemented with minimal essential medium containing antibiotics and glutamine. After 8 h the medium was removed and replaced with 0.5 ml of a 1:20 dilution of filter-sterilized test serum in medium containing 5% fetal calf serum and also containing a 1:50 dilution of rabbit anti-RSV G glycoprotein sera (neutralization titer of 1:5,000). In the presence of G antiserum, the virus can spread only by direct cell-to-cell spread, which is mediated by the F glycoprotein. After 40 h the medium was removed, cover slips were washed once with phosphate-buffered saline, air-dried, and acetone-fixed, and the size and number of infected foci were visualized by using indirect immunofluorescence. A monoclonal antibody (D14) to the nucleocapsid protein was then added at a dilution of 1:500 of ascites fluid, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:1,000) was added. The foci were scored on a scale of 0 to 4 as follows: 0, many large syncytia, indicating the absence of FI activity; 1+, many single or double infected cells and many small syncytia; 2+, primarily single or double infected cells with a few small syncytia; 3+, only single infected cells or two adjacent double infected cells; and 4+, only single infected cells, indicating a high level of FI antibody activity.

The correlation of the ELISA-F serum antibody titer and level of FI activity is presented using preimmunization sera for analysis of maternally derived antibody (Fig. 1A) as well as acute- and convalescent-phase sera from unimmunized infants and children (Fig. 1B). There was a highly significant correlation between FI and ELISA antibody levels for each set of sera. These data indicate that F antibodies raised during primary infection (Fig. 1B) or following repeated infection (Fig. 1A, maternally derived antibody) have a similar amount of ELISA-F binding activity and FI activity.

The relationship of ELISA-F binding antibody to FI activity in the sera of the RSV vaccinees was investigated next (Table 1). The preimmune sera of the RSV vaccinees were collected when the infants possessed a high level of maternally derived antibodies, which declined to a lower level 1 month postimmunization. The 16-fold rise in titer of ELISA antibody in the RSV vaccinees between the samples taken 1 month after the first dose and 1 month after the third dose indicates that the Formalin-inactivated RSV vaccine was immunogenic. The ELISA-F titers achieved in the vaccinees and in the infected infants and children were similar. In contrast, the level of postimmunization FI antibody activity of the RSV vaccinees was significantly less than that achieved in the postinfection sera of the infected infants and children who had not received the RSV vaccine. The FI and ELISA-F responses of the individual infants and children are shown in Fig. 2. The data (Table 1 and Fig. 2) indicate that the postimmunization sera of the RSV vaccinees are deficient in FI antibody activity despite the presence of moderate levels of ELISA-F-binding antibodies.

The present data in the context of our previous findings suggest that epitopes on the F glycoprotein involved in inducing neutralizing and FI antibodies were modified by

TABLE 1. ELISA-F and FI antibody responses of infants and children immunized with Formalin-inactivated RSV or infected naturally with RSV[a]

Group	No. in group	FI antibody activity (mean ± SE)			ELISA-F antibody titer (reciprocal mean $\log_2$ ± SE)		
		Pre or acute	Post 1	Post 3 or convalescent[b]	Pre or acute	Post 1	Post 3 or convalescent[c]
RSV vaccinees	21	1.9 ± 0.4	0.3 ± 0.2	0.6 ± 0.2	14.0 ± 0.9	9.8 ± 0.5	13.6 ± 0.3
Natural infection	15	0.3 ± 0.2	NA[d]	2.5 ± 0.3	7.4 ± 0.6	NA	12.9 ± 0.3

[a] The ELISA detected IgG antibody to the F glycoprotein (6). A complete set of sera from the RSV vaccinees was not available for each child. The mean levels of antibody present were determined for 19 to 21 of the vaccinees. Serum samples were obtained from vaccinees before immunization (pre), 1 month following the first dose of vaccine (post 1), and 1 month following the third dose (post 3) of vaccine. RSV infections did not occur during this time period in these vaccinees. Acute- and convalescent-phase serum samples were obtained from subjects with natural infections.

[b] Values significantly different at $P < 0.001$.

[c] Values not significantly different ($P > 0.10$).

[d] NA, Not applicable.

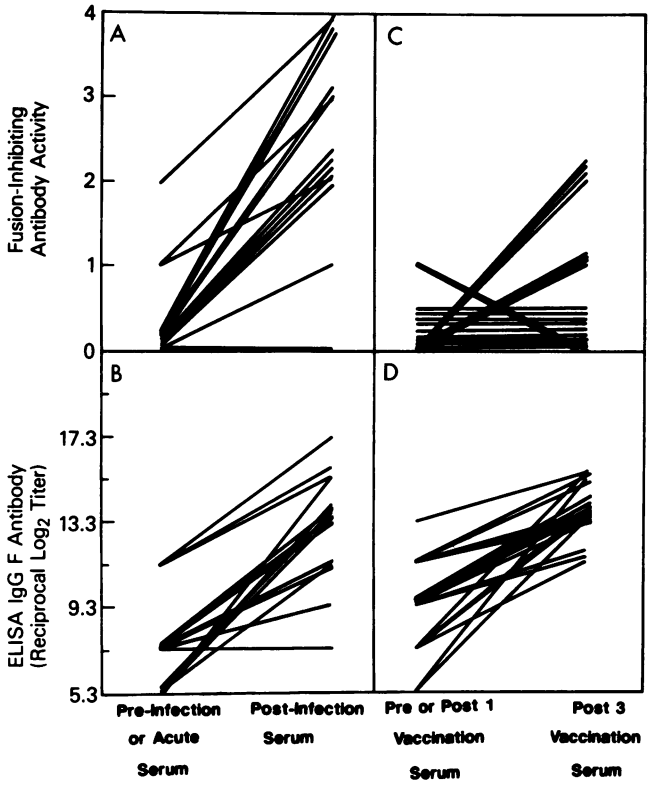

FIG. 2. ELISA and FI antibody responses of individual infants and children to natural infection (A and B) immunization with Formalin-inactivated RSV vaccine (C and D). The first serum sample from the RSV vaccinees was either the preimmunization (pre) or 1-month post-first vaccination (post 1) sample, whichever had the lower titer. This was done because it was noted that many children had a decline in antibody titer 1 month postvaccination resulting from a decline in maternally derived antibodies and a poor response to vaccine. Postimmunization serum 3 (post 3) was obtained 1 month following the third immunization.

Formalin, resulting in reduction of a functional antibody response, whereas nonneutralization and non-FI epitopes were relatively unaltered. Thus, Formalin-treated RSV stimulated and unbalanced immune response in which an unusually large proportion of the induced antibodies were directed against nonprotective epitopes on the F as well as the G glycoprotein (7). Consequently, effective resistance was not provided, but a high concentration of antibodies that bind to RSV proteins, such as the F and G glycoproteins, were present in the serum and were available to form complexes with viral antigens that were produced during infection. The formation of immune complexes in the lungs could have played a part in the enhanced pulmonary disease in the Formalin-inactivated RSV vaccinees and in immunized and challenged cotton rats (9). New RSV vaccines that are currently being developed should induce a more balanced antibody response than that induced by Formalin-inactivated RSV vaccine.

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