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Dissociation between Serum Neutralizing and Glycoprotein Antibody Responses of Infants and Children Who Received Inactivated Respiratory Syncytial Virus Vaccine

BRIAN R. MURPHY,[1]* GREGORY A. PRINCE,[1] EDWARD E. WALSH,[2] HYUN WHA KIM,[3] ROBERT H. PARROTT,[3] VAL G. HEMMING,[4] WILLIAM J. RODRIGUEZ,[3] AND ROBERT M. CHANOCK[1]

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[1]; Department of Medicine, Infectious Diseases Unit, Rochester General Hospital, Rochester, New York 14621[2]; Children's Hospital National Medical Center and George Washington University, Washington, D.C. 20010[3]; and Department of Pediatrics, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814[4]

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The serum antibody response of infants and children immunized with Formalin-inactivated respiratory syncytial virus (RSV) vaccine 20 years ago was determined by using an enzyme-linked immunosorbent assay specific for the RSV fusion (F) and large (G) glycoproteins and a neutralization assay. Twenty-one young infants (2 to 6 months of age) developed a high titer of antibodies to the F glycoprotein but had a poor response to the G glycoprotein. Fifteen older individuals (7 to 40 months of age) developed titers of F and G antibodies comparable to those in children who were infected with RSV. However, both immunized infants and children developed a lower level of neutralizing antibodies than did individuals of comparable age with natural RSV infections. Thus, the treatment of RSV with Formalin appears to have altered the epitopes of the F or G glycoproteins or both that stimulate neutralizing antibodies, with the result that the immune response consisted largely of "nonfunctional" (i.e., nonneutralizing) antibodies. Subsequent natural infection of the vaccinees with wild-type RSV resulted in enhanced pulmonary disease. Despite this potentiation of illness, the infected vaccinees developed relatively poor G, F, and neutralizing antibody responses. Any or all of three factors may have contributed to the enhancement of disease in the RSV-infected vaccinees. First, nonfunctional antibodies induced by the inactivated RSV vaccine may have participated in a pulmonary Arthus reaction during RSV infection. Second, the poor antibody response of infants to the G glycoprotein present in the Formalin-inactivated vaccine may have been inadequate to provide effective resistance to subsequent wild-type virus infection. Third, the relatively reduced neutralizing antibody response of the infant vaccinees to wild-type RSV infection may have contributed to their enhanced disease by delaying the clearance of virus from their lungs.

Respiratory syncytial virus (RSV), which is classified in the pneumovirus subgroup of the *Paramyxoviridae* family (8), is the leading cause of severe lower-respiratory-tract disease in infancy (5, 15). Thus far, attempts to develop a live or inactivated vaccine effective against RSV have been unsuccessful (3, 4, 6, 22). Approximately 20 years ago a Formalin-inactivated RSV vaccine was evaluated in infants and young children. 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 (6). 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 (3, 4, 6).

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 (17). 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%, as compared with that in control animals. An examination of the immunogenicity of the Formalin-inactivated RSV vaccine in cotton rats revealed that these animals developed levels of enzyme-linked immunosorbent assay (ELISA) antibodies to the large (G) and fusion (F) surface glycoproteins of RSV comparable to those attained in animals with pulmonary or systemic infections. However, the vaccinated cotton rats developed a level of neutralizing antibodies that was only 1/30 that in animals infected with RSV. Because the G and F glycoproteins are the major antigens to which neutralizing antibodies are directed (19–21), it appeared that most of the antibodies against these glycoproteins produced by the vaccinated animals lacked functional, i.e., neutralizing, activity. Thus, Formalin treatment of RSV appears preferentially to alter the surface glycoprotein epitopes that stimulate neutralizing antibodies. In the present study, we examined the sera of infants and children inoculated 20 years ago with Formalin-inactivated RSV vaccine to determine if their neutralizing antibody response as well as their antibody responses to RSV G and F glycoproteins resembled those previously observed for cotton rats inoculated with the same vaccine (17). Our findings indicate that in humans, as well as cotton rats, Formalin-inactivated RSV induces antibodies to the RSV surface glycoproteins which have a reduced capacity to neutralize the virus.

MATERIALS AND METHODS

Study groups. Infants between 2 and 7 months of age (median age, 3.5 months) who attended a Child Health

* Corresponding author.

TABLE 1. Serum antibody responses of infants and children to inactivated RSV vaccine

Group (age [mo] at time of start of immunization or time of infection)	No. in group	Assay for antibody response[a]	Mean titer of IgG antibody (reciprocal $\log_{10}$)				
			Preinjection or acute	1 mo after first injection	1 mo after second injection	Prior to third injection	3 weeks after third injection or postinfection
CHC vaccinees[b] (2 to 7)	21	ELISA F	4.2 ± 0.3	3.0 ± 0.1	3.1 ± 0.1	3.6 ± 0.1	4.1 ± 0.1
		ELISA G	3.6 ± 0.2	3.0 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	2.7 ± 0.1
		Neutralization	2.4 ± 0.2	1.4 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
JV vaccinees[b] (7 to 40)	15	ELISA F	3.1 ± 0.2				5.0 ± 0.2
		ELISA G	2.2 ± 0.3				3.6 ± 0.1
		Neutralization	1.6 ± 0.1				2.2 ± 0.1
Natural infection (1 to 8)	11	ELISA F	2.9 ± 0.2				3.5 ± 0.3
		ELISA G	2.8 ± 0.3				3.5 ± 0.1
		Neutralization	1.7 ± 0.2				2.4 ± 0.2
Natural infection (9 to 24)	13	ELISA F	2.3 ± 0.2				4.2 ± 0.1
		ELISA G	2.2 ± 0.2				3.7 ± 0.1
		Neutralization	1.2 ± 0.1				2.8 ± 0.1
PIV1[c] vaccinees (2 to 7)	9	ELISA F	2.7 ± 0.2	2.5 ± 0.1	2.3 ± 0.2	2.1 ± 0.1	2.1 ± 0.3
		ELISA G	3.1 ± 0.1	3.0 ± 0.1	2.8 ± 0.1	2.4 ± 0.1	2.3 ± 0.1
		Neutralization	1.5 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.2

Center at the Children's Hospital National Medical Center in Washington, D.C., were selected for administration of vaccine after parental consent was obtained (CHC group) (6). A second group of vaccinees, 7 to 40 months of age (median age, 22.2 months), were residents of Junior Village (JV group) (4). A third group of 13 infants and children who were hospitalized at Children's Hospital National Medical Center with RSV bronchiolitis or pneumonia in the late 1960s ranged in age from 4 to 21 months (median age, 10.2 months) (5, 15). A fourth group of 11 infants and children ranging in age from 1 to 14 months (median age, 5.2 months) were hospitalized at Children's Hospital National Medical Center with RSV bronchiolitis or pneumonia or both during the winter of 1984 to 1985 and participated in a double-blind, placebo-controlled study designed to evaluate the therapeutic efficacy of intravenous gamma globulin. Only infants and children who had received the placebo were included in the present study.

Administration of vaccine and collection of serum specimens. The Formalin-inactivated RSV vaccine (lot 100) and the schedule for its administration were described in detail previously and will only be summarized here (4, 6). The RSV vaccine contained Formalin-inactivated, alum-precipitated, 100-times-concentrated virus (Bernett strain) grown in a vervet monkey kidney cell culture. A parainfluenza virus type 1 (PIV1) vaccine was prepared in the same manner and served as a control in the evaluation of the lot 100 RSV vaccine. Two 0.5-ml intramuscular injections of RSV or PIV1 vaccine were given 1 month apart, and a third injection was given 3 months later. Serum for the antibody assay was collected from the CHC group before the first and second doses of vaccine, 1 month after the second dose of vaccine, and before and 3 weeks after the third dose of vaccine. In addition, blood was collected at the time of and 1 month after community-acquired RSV illness. A complete set of sera from the CHC group was not available for each of the 21 vaccinees, but the smallest proportion tested at any interval was 80%. Only the pre-first-dose and post-third-dose sera were available from the JV group.

Neutralization assay and ELISA. Serum neutralizing antibody titers were measured by a plaque reduction, complement-enhanced assay as previously described using a 60% plaque reduction endpoint (10). The A2 strain of RSV was used in the neutralization assay; this strain is closely related to the Bernett strain (also known as strain 11657) that was present in the lot 100 RSV vaccine (2).

Antibodies to the G and F glycoproteins were measured with an ELISA that used a highly purified preparation of G or F glycoprotein isolated by immunoaffinity chromatography as previously described (11, 19, 21). The Long strain of RSV, which was used to produce the G and F ELISA antigens, is closely related to the Bernett strain used to prepare the vaccine (2).

RESULTS

Response of infants and children to vaccination with RSV vaccine. The serum antibody responses of the CHC (younger) and JV (older) RSV vaccinees were determined and compared with those of infants and children with natural RSV infections (Table 1). The response of the CHC vaccinees was most like that of infants 1 to 8 months of age, while the response of the JV vaccinees was most like that of the older infants and young children. Comparison with these age-matched groups was necessary because the antibody response of infants 1 to 8 months of age to RSV infection is significantly reduced in comparison to that of older infants and young children (11). The antibody responses to the F and G glycoproteins were measured for both the immunoglobulin G (IgG) and IgA isotypes because the IgA assay is slightly more efficient for the detection of a seroresponse in individuals with a high level of maternally derived IgG antibody, the usual circumstance of younger infants (11). In this analysis we included only individuals who received the complete schedule of three doses of vaccine and who were not infected with RSV during the course of immunization.

Ninety-five percent of the CHC vaccinees developed a response to the F glycoprotein, and the titer achieved was

TABLE 1—*Continued*

% with ≧4-fold rise in IgG antibody titer	Mean titer of IgA antibody (reciprocal $\log_{10}$)					% with ≧4-fold rise in IgA antibody titer	% with ELISA IgG or IgA rise in titer or both	Ratio ($\log_{10}$) of post vaccination or postinfection IgG F antibody titer to neutralizing antibody titer
	Preinjection	1 mo after first injection	1 mo after second injection	Prior to third injection	3 weeks after third injection or postinfection			
95	1.5 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.8 ± 0.1	81	95	2.4
33	1.8 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	33	43	
48								
100	2.6 ± 0.2				3.5 ± 0.2	87	100	2.8
93	1.9 ± 0.3				2.6 ± 0.2	60	93	
60								
54	1.2 ± 0.1				1.9 ± 0.2	82	82	1.1
64	1.2 ± 0.1				2.2 ± 0.3	73	73	
64								
92	1.4 ± 0.1				2.7 ± 0.2	92	100	1.4
92	1.1 ± 0.1				2.0 ± 0.2	62	92	
92								
0	1.4 ± 0.3	1.2 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	0	0	1.1
0	1.3 ± 0.3	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	0	0	1.0
0								

[a] Neutralizing antibody titers are shown only for IgG antibody because neutralizing activity in preimmunization specimens reflects maternally derived IgG antibody.
[b] Formalin-inactivated RSV vaccine (lot 100).
[c] Formalin-inactivated PIV1 vaccine.

slightly greater than that in age-matched, unvaccinated patients with RSV lower-respiratory-tract illness. Similar observations were made for the JV vaccinees. These results suggest that the F component of the RSV vaccine was highly immunogenic. In contrast, only 43% of the younger vaccinees developed a G antibody response. However, 93% of the older vaccinees developed a G antibody response, with a mean titer of G antibodies comparable to that in the age-matched controls (age, 9 to 24 months), who had naturally acquired RSV disease. Thus, the G component of the RSV vaccine was also immunogenic, but maximal immunogenicity was expressed only in the older vaccinees, i.e., 9 to 24 months of age. The mean titer of G antibodies (IgG isotype) in the postimmunization serum specimens from CHC vaccinees was one-eighth that in age-matched patients with RSV disease. However, the prevaccination ELISA IgG G antibody titer in the CHC vaccinees was significantly higher than that in acute-phase sera from age-matched controls as well as that in the JV vaccinees, and this high level of transplacentally acquired antibodies may have suppressed the response of the CHC group to the G component of the Formalin-inactivated vaccine. Although the JV vaccinees were selected to have a low titer of prevaccination RSV antibodies, we estimate that about 60% were infected previously with RSV, in which case vaccination would be expected to induce a secondary response to the F or G glycoprotein or both. For this reason, caution should be exercised in interpreting the responses of these vaccinees.

The neutralizing antibody response of the CHC vaccinees was significantly lower than that of the unvaccinated individuals of comparable age with natural RSV infection, despite the fact that both groups of individuals developed a comparable, high-level F antibody response. The JV vaccinees also developed a relatively low titer of neutralizing antibodies in comparison with age-matched controls with RSV disease, even though immunization induced a significantly higher titer of ELISA F antibody ($P < 0.01$, Student's t test).

The ELISA F antibody titer/neutralizing antibody titer ratios for the vaccinees were 250 (CHC group) and 640 (JV group), about 25-fold higher than those of the appropriate comparison groups. Previously, it was observed that the ELISA F antibody titer/neutralizing antibody titer ratio for RSV-vaccinated cotton rats was 62-fold higher than that for unvaccinated, RSV-infected animals. A similar analysis for the G glycoprotein was not performed because of the poor response of the CHC vaccinees to this antigen.

Immune response of CHC vaccinees to natural infection with RSV. Previously it was observed that Formalin-inactivated RSV vaccine did not protect against infection and, when infection occurred, vaccinees developed a disease of increased severity (6). We examined the immune response of these vaccinees to subsequent natural infection with RSV (Table 2). Following RSV infection, the vaccinees had reduced serum ELISA F and G antibody responses as well as a reduced neutralizing antibody response in comparison with age-matched PIV1 vaccine controls or unvaccinated patients with RSV disease.

DISCUSSION

A comparison of the humoral immune responses of infants and children to immunization with inactivated measles virus or RSV vaccine is helpful for understanding the mechanism(s) underlying the enhancement of disease following infection by the respective wild-type virus (3, 18). Formalin inactivation of measles virus, also a member of the *Paramyxoviridae* family, led to the preferential inactivation of the antigen that stimulates hemolysin-inhibiting antibodies, i.e., antibodies directed against the F (fusion) protein that mediates lysis of erythrocytes, virus penetration, and syncytium formation (12). This preferential inactivation of

TABLE 2. Serum antibody responses of RSV vaccinees to natural infection with RSV

Individuals infected with RSV	No. in group	Assay for antibody response	Titer of antibody (reciprocal $\log_{10}$) of the indicated isotype:					
			IgG			IgA		
			Preinfection	Postinfection	% with fourfold rise	Preinfection	Postinfection	% with fourfold rise
RSV vaccinees[a]	12	ELISA F	4.1 ± 0.1[b]	4.4 ± 0.1	33	1.8 ± 0.2	2.0 ± 0.2	17
		ELISA G	2.8 ± 0.2[b]	3.1 ± 0.3	25	1.4 ± 0.2	1.7 ± 0.3	25
		Neutralization	1.7 ± 0.2[b]	2.0 ± 0.2	17			
PIV1 vaccinees	4	ELISA F	2.2 ± 0.4	3.9 ± 0.3	100	1.9 ± 0.3	3.1 ± 0.2	100
		ESISA G	2.2 ± 0.2	4.3 ± 0.3	100	1.6 ± 0.2	2.8 ± 0.2	100
		Neutralization	1.0 ± 0.0	2.6 ± 0.2	100			
Unvaccinated[c] (9 to 24 months old)	13	ELISA F	2.3 ± 0.2	4.2 ± 0.1	92	1.4 ± 0.01	2.7 ± 0.2	92
		ELISA G	2.2 ± 0.2	3.7 ± 0.1	92	1.1 ± 0.1	2.0 ± 0.2	62
		Neutralization	1.2 ± 0.1	2.8 ± 0.1	92			

[a] Six of the RSV vaccinees were hospitalized with RSV bronchiolitis or pneumonia or both, and three of the remaining vaccinees developed RSV pneumonia or bronchiolitis or both not requiring hospitalization.

[b] Preinfection sera were not available from three of the RSV vaccinees. Instead, postvaccination sera obtained 3 weeks after the last vaccination were used in this analysis. Each of the RSV vaccinees was infected within 1 year after receiving the full three-inoculation course of immunization.

[c] Each of the 13 individuals was hospitalized, 8 with bronchiolitis and 5 with pneumonia.

the F antigen by Formalin treatment appears to be a general characteristic of paramyxoviruses (13, 14). The vaccinees who received Formalin-inactivated measles virus vaccine developed hemagglutination-inhibiting antibodies that neutralized infectivity, but antibodies to the F protein were not detected by the functional hemolysin-inhibiting antibody assay (12). These vaccinees were protected for several years following immunization until their serum hemagglutination-inhibiting (neutralizing) antibody titers fell to a low or undetectable level, at which time infection of some vaccinees with wild-type measles virus led to a severe pneumonia not characteristic of natural infections (18). Exaggerated local reactions to live measles virus vaccine given subcutaneously were also observed at a time when the serum hemagglutination-inhibiting antibody titers of the vaccinees had decreased to a low level. Biopsy of the resulting skin lesions revealed infiltration of neutrophils and deposition of IgG, complement, and measles antigen in blood vessel walls, suggesting the occurrence of an Arthus reaction (1). It was subsequently suggested that the severe pneumonia seen in the vaccinees who received inactivated measles virus might be a form of pulmonary Arthus reaction (9). In this reaction, the measles virus antigen produced during virus replication in pulmonary tissue reacts with antibody induced during the secondary immune response characteristic of inactivated-virus vaccinees naturally infected with measles virus (9).

Our observations on the responses of cotton rats and humans to Formalin-inactivated RSV vaccine also suggest that the severe bronchiolitis and pneumonia that developed when the RSV vaccinees were infected with RSV were the result of a pulmonary Arthus reaction. This hypothesis is based upon the following observations. Formalin treatment of RSV selectively altered the epitopes of the F or G glycoprotein or both that stimulate neutralizing antibodies (17). Furthermore, parenteral inoculation of cotton rats with the Formalin-inactivated vaccine failed to elicit detectable levels of antibodies that prevented syncytium formation, whereas immunization with purified F glycoprotein did induce such antibodies (17, 19). The net result of inoculation of cotton rats (and presumably humans) with Formalin-inactivated vaccine was stimulation of a high level of ELISA F- or G-binding antibodies or both that were largely devoid of neutralizing and syncytium-inhibiting activities; in other words, high levels of "nonfunctional" antibodies were induced. It is not known whether nonfunctional antibodies to the F protein were induced for measles virus, but the available evidence indicates that the neutralizing and hemolysin-inhibiting activities associated with the F antigen were not stimulated by Formalin-inactivated measles virus (12). The poor response of the young CHC vaccinees to the G glycoprotein may also have contributed to their lack of resistance to infection. This diminished response may well have been due to an immunosuppressive effect of maternal G antibodies on the production of such antibodies by young infants (B. R. Murphy, D. W. Alling, M. H. Snyder, E. E. Walsh, G. A. Prince, R. M. Chanock, V. G. Hemming, W. J. Rodriguez, H.-W. Kim, and P. F. Wright, unpublished observations). Consistent with this suggestion were the high-level ELISA IgG G antibody responses of older JV vaccinees and seronegative adult cotton rats to immunization with Formalin-inactivated RSV vaccine. The level of neutralizing antibodies that the Formalin-inactivated vaccine induced in the CHC vaccinees was considerably below that required to prevent RSV infection of the lungs of cotton rats (16). We suggest that when the CHC vaccinees were infected with wild-type RSV, virus replicated in the bronchiolar epithelium, and the antigens that were produced reacted with the predominantly nonfunctional (nonneutralizing) antibodies that had been induced by the vaccine. In this manner a local Arthus reaction occurred at the sites of RSV replication in the bronchioles and alveoli, resulting in an enhancement of pulmonary pathology. Infection could spread and easily involve other cells because vaccine-induced F and G antibodies would have a diminished capacity to neutralize infectivity and, in the case of F antibodies, there would be a decreased capacity to prevent the direct spread of virus from cell to cell. In this manner additional rounds of RSV replication would occur, the antigen-antibody complexes generated would intensify the Arthus reaction, and unusually severe bronchiolitis or pneumonia or both would develop. Two observations that emerged from studies in cotton rats are consistent with this hypothesis (17). First, the initial infiltrate seen in the lungs of immunized cotton rats challenged with RSV consists primarily of neutrophils, an observation consistent with the occurrence of an

Arthus reaction. This infiltrate resembled the neutrophilic infiltrate seen in the skin biopsies of children immunized with inactivated measles virus vaccine and subsequently challenged subcutaneously with live measles virus (1). Second, the intermediate level of RSV replication achieved in the lungs of previously immunized, infected cotton rats is consistent with the spread of virus in the presence of nonfunctional antibodies (17).

The finding that RSV vaccinees who were infected with wild-type RSV had poor G, F, and neutralizing antibody responses was unexpected. In contrast, vaccinees who received inactivated measles virus had a high-level response to the hemagglutinin protein and a moderate response to the F protein of measles virus, with the development of both neutralizing and hemolysin-inhibiting antibodies (12). However, measles virus and RSV vaccinees differed in that the former had low titers of both F and hemagglutination-inhibiting antibodies at the time of natural virus infection, whereas the latter had a high titer of F antibodies. The young vaccinees who had a high titer of vaccine-induced F antibodies developed relatively poor F and neutralizing antibody responses to subsequent RSV infections. This result may have contributed to their enhanced disease by delaying the clearance of virus from lungs during infection. The mechanism(s) underlying the decreased responsiveness of RSV vaccinees to wild-type virus infections, however, remains to be elucidated. We have recently observed that immunized cotton rats also have a blunted immune response to wild-type RSV infections (G. A. Prince and B. R. Murphy, unpublished observations). It is important to add that although RSV did replicate in immunized cotton rats following challenge, the level of replication was reduced 95% in comparison with that in uninfected controls. It is reasonable to suggest that diminished replication of RSV may have contributed to the poor immune response to infection of human vaccinees.

Although we have focused on the possibility that enhanced disease was due to a pulmonary Arthus reaction, it is possible that other forms of immunologic injury, such as a pulmonary delayed-type hypersensitivity reaction, could have been involved. In the lungs of immunized cotton rats challenged with RSV a lymphocytic infiltrate appears after the initial neutrophilic infiltrate, an observation consistent with the occurrence of a delayed-type hypersensitivity reaction (17). Furthermore, infants and children who received Formalin-inactivated vaccine had an in vitro lymphoproliferative response to RSV antigens that was significantly greater than that in individuals naturally infected with RSV (7). These observations suggest that the enhanced disease seen in the RSV vaccinees may have at least two immunologic components, a humorally mediated Arthus reaction and a cellularly mediated delayed-type hypersensitivity reaction. We are currently investigating the effect of Formalin treatment on the ability of isolated RSV G and F glycoproteins to stimulate antibodies and to enhance pathology during infection of immunized cotton rats. The hallmarks of Arthus reactions, i.e., IgG, complement, and antigen, are being sought in the lungs of immunized, RSV-infected cotton rats. Transfer of cells and serum obtained from immunized cotton rats to histocompatible inbred cotton rats should enable us to further determine the relative contribution of antibodies and cells to vaccine potentiation. These planned studies in cotton rats should provide additional insight into the mechanisms of the enhanced disease observed previously in the RSV vaccinees and should provide a more secure basis for evaluating new approaches to RSV immunoprophylaxis. The present study demonstrates a similarity between the serological responses of humans and cotton rats to the Formalin-inactivated RSV vaccine and gives additional credence to the study of RSV in cotton rats, the most permissive small experimental animal.

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