JOURNAL OF VIROLOGY, Oct. 1987, p. 3163–3166
0022-538X/87/103163-04$02.00/0

Vol. 61, No. 10

Antigenic Relatedness between Glycoproteins of Human Respiratory Syncytial Virus Subgroups A and B: Evaluation of the Contributions of F and G Glycoproteins to Immunity

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Received 9 February 1987/Accepted 16 June 1987

The degree of antigenic relatedness between human respiratory syncytial virus (RSV) subgroups A and B was estimated from antibody responses induced in cotton rats by respiratory tract infection with RSV. Glycoprotein-specific enzyme-linked immunosorbent assays of antibody responses induced by RSV infection demonstrated that the F glycoproteins of subgroups A and B were antigenically closely related (relatedness, $\hat{R}$ ~ 50%), whereas the G glycoproteins were only distantly related ($\hat{R}$ ~ 5%). Intermediate levels of antigenic relatedness ($\hat{R}$ ~ 25%) were seen in neutralizing antibodies from cotton rats infected with RSV of the two subgroups. Immunity against the F glycoprotein of subgroup A, induced by vaccinia-A2-F, conferred a high level of protection which was of comparable magnitude against challenge by RSV of either subgroup. In comparison, immunity against the G glycoprotein of subgroup A, induced by vaccinia-A2-G, conferred less complete, but significant, protection. Importantly, in vaccinia-A2-G-immunized animals, suppression of homologous challenge virus replication was significantly greater (13-fold) than that observed for the heterologous virus.

Since its initial isolation from humans in 1956 (6), human respiratory syncytial virus (RSV) has become recognized as the major respiratory tract pathogen of infants and young children. Two major antigenic subgroups of RSV have been described (3, 7, 8, 11, 14). These two subgroups, designated A and B, have been segregated on the basis of in vitro neutralization assays (7, 8) and reactivity patterns with panels of monoclonal antibodies, with the greatest divergence observed for G, the putative attachment glycoprotein (3, 11, 14).

The importance of antigenic variation among RSV strains has remained unclear. Experimental infection of the respiratory tract of cotton rats with RSV representing either subgroup induced resistance to challenge with homologous or heterologous virus (17). However, several investigators have recently found that subgroup A and B viruses can circulate separately or concurrently during RSV epidemics, which raises the possibility that RSV antigenic heterogeneity may play a role in the incomplete immunity observed after RSV infection (1, 13).

Recent advances in the molecular biology of RSV (9 and references cited therein) have provided new reagents for characterizing host immunity to RSV. The availability of purified RSV envelope glycoproteins for use in immunoassays (21, 22) and of recombinant vaccinia virus vectors that express individual RSV envelope glycoproteins (5, 10, 16, 20) have allowed a comparison of the individual contributions of the F and G glycoproteins to host immunity (16). In this study, we have used these reagents to (i) estimate the degree of antigenic relatedness of the F and G glycoproteins between subgroups A and B, (ii) quantitate heterologous antibody responses in cotton rats given vaccinia virus-RSV (subgroup A) recombinants, and (iii) assess the ability of the vaccinia virus recombinants to protect against infection with a heterologous (subgroup B) virus.

MATERIALS AND METHODS

Cells and viruses. The vaccinia virus recombinants used in this study were constructed previously (10, 16). The designations are as follows. Vaccinia-A2-F and vaccinia-A2-G are vaccinia virus (WR strain)-RSV recombinants expressing the F and G glycoproteins, respectively, of the RSV A2 strain. Vaccinia-βgal is a vaccinia virus recombinant that expresses β-galactosidase and was used as a negative control for immunizations. Three RSV strains were plaque purified and propagated in HEp-2 cell monolayers; strains A2 and Long are subgroup A viruses, and strain 18537 is a subgroup B virus (3, 14). The correct subgroup identifications and the purity for these specific virus stocks were kindly confirmed by R. M. Hendry on the basis of monoclonal antibody reactivity patterns.

Serum antibody assays. Antibody responses to the RSV F and G glycoproteins were measured by enzyme-linked immunosorbent assay (ELISA), using immunoaffinity-purified F and G glycoproteins (from strains Long and 18537) as solid-phase antigens (15). The Long strain G protein was kindly provided by Peter Paradiso and Steve Hildreth, Praxis Biologics, Rochester, N.Y. RSV serum-neutralizing antibody titers were determined by a 60% plaque reduction assay (7).

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TABLE 1. Homologous and heterologous RSV antibody responses at 21 days after primary immunization

Primary immunization[a] with:	No. of animals	Titer of serum ELISA antibodies reactive with indicated purified glycoprotein of[b]:				Titer of serum-neutralizing antibodies against:	
		Subgroup A (strain Long)		Subgroup B (strain 18537)		Subgroup A (strain A2)	Subgroup B (strain 18537)
		F	G	F	G		
None	10	3.8 ± 0.6	3.3 ± 0	3.5 ± 0.2	3.5 ± 0.2	4.3 ± 0	4.3 ± 0
RSV A2	10	16.3 ± 0.4	12.9 ± 0.4	14.1 ± 0.4	9.9 ± 0.7	11.2 ± 0.5	9.9 ± 0.5
RSV Long	9	15.1 ± 0.4	13.3 ± 0.7	10.9 ± 1.1	10.0 ± 0.7	9.8 ± 0.6	8.1 ± 0.4
RSV 18537	8	14.3 ± 0.8	6.8 ± 0.8	13.3 ± 0.5	12.1 ± 0.4	6.9 ± 0.6	9.3 ± 0.4
Vaccinia-βgal	17	4.8 ± 0.4	3.7 ± 0.2	4.1 ± 0.3	4.5 ± 0.3	4.4 ± 0.1	4.3 ± 0.0
Vaccinia-A2-F	19	18.5 ± 0.4	5.7 ± 0.6	16.1 ± 0.4	5.1 ± 0.5	12.3 ± 0.4	12.5 ± 0.5
Vaccinia-A2-G	20	4.0 ± 0.2	15.9 ± 0.3	3.8 ± 0.2	13.9 ± 0.4	11.1 ± 0.5	9.0 ± 0.5

[a] The dose of RSV was 10^5 PFU given intranasally. The dose of vaccinia virus recombinants was 10^8 PFU given intradermally.
[b] Antibody titers are expressed as the geometric mean ($\log_2$).

Animal studies. Young adult cotton rats (*Sigmodon hispidus*) were immunized by intradermal inoculation with vaccinia-βgal, vaccinia-A2-F, or vaccinia-A2-G (10^8 PFU in a 0.1-ml inoculum). Other rats were immunized by intranasal inoculation with RSV strain A2, Long, or 18537 (10^5 PFU in a 0.1-ml inoculum). At 3 weeks after immunization, animals were bled and challenged with the A2 or 18537 RSV strain ($10^{5.4}$ PFU inoculated intranasally). Animals were sacrificed at 4 days after challenge, and RSV titers in nasal turbinates and lung homogenates were measured by plaque titration (18).

RESULTS

Serologic responses to primary immunization. Homologous and heterologous antibody responses induced in cotton rats were measured at 21 days after primary immunization by glycoprotein-specific ELISA and in vitro neutralization assays (Table 1). ELISA antibody titers to homologous and heterologous F glycoproteins were generally similar; the largest difference was observed in animals immunized with RSV Long strain. In contrast, the heterologous antibody responses to the G glycoproteins were quite distinct (Table 1). Animals infected with subgroup B (strain 18537) virus had a 40-fold higher titer to homologous G glycoprotein than to heterologous G glycoprotein. Similarly, cotton rats infected with a subgroup A (strain A2 or Long) virus showed an eightfold-higher titer against homologous G glycoprotein. Thus, the high degree of antigenic variation in the G glycoproteins detected by monoclonal antibody reactivity also was observed with polyclonal antibodies induced by respiratory tract infection.

The degree of antibody cross-reactivity in animals infected with a subgroup A or B virus was estimated by use of the Archetti-Horsfall formula (4), and the results are presented in Table 2. The Long and A2 strains were used interchangeably since both are subgroup A viruses. Since antibody responses to the F glycoproteins were similar among the three RSV strains, there was no significant difference in relatedness between any of the three possible pairs of strains. On the other hand, responses to the G glycoproteins varied up to 40-fold, and there was a highly significant difference in relatedness between each pair of strains that belonged to different subgroups.

Animals that received vaccinia-A2-F or vaccinia-A2-G developed very high levels of antigen-specific ELISA antibodies (Table 1). When compared with RSV infection of the respiratory tract, infection with the vaccinia virus-RSV recombinants elicited a greater (fourfold or more) ELISA antibody response to the surface glycoproteins of both RSV subgroups. For both recombinants, antibody titers to homologous glycoproteins were slightly higher than to heterologous glycoproteins. However, these differences could not be analyzed with the Archetti-Horsfall method since vaccinia virus-RSV 18537 recombinants were not available for reciprocal immunizations.

These observations of the degree of relatedness between the F and G glycoproteins correlated with cross-subgroup in vitro neutralization responses induced by the vaccinia virus-RSV A2 recombinants (Table 1). Sera from animals inoculated with vaccinia-A2-F neutralized both subgroup A and B viruses with equal efficiency, a result consistent with the significant antigenic relatedness of the F glycoproteins between the subgroups, whereas sera from animals given vaccinia-A2-G neutralized the homologous A2 virus more efficiently (fourfold) than the heterologous 18537 virus.

Sera from the animals infected intranasally with RSV also differed in the ability to neutralize the homologous or heterologous subgroup virus in vitro (Table 1). Neutralization titers for homologous virus were 2.5- to 5-fold higher than

TABLE 2. Relatedness between RSV strains as measured by ELISA and neutralization assays

Antibody assay and strain comparison[a]	Percent relatedness ($\hat{r}$)[b]	SD[c]	*P* value
ELISA anti-F			
Long vs 18537	33 (−3.2)	1.94	>0.10
A2 vs 18537	66 (−1.2)	1.67	>0.20
ELISA anti-G			
Long vs 18537	5.1 (−8.6)	1.94	<0.001
A2 vs 18537	5.7 (−8.3)	1.67	<0.001
Neutralization			
Long vs 18537	25 (−4.0)	1.23	<0.01
A2 vs 18537	28 (−3.7)	1.22	<0.01

[a] $\log_2$ titers (see Table 1) were converted to arithmetic titers for calculations.
[b] Calculated by using the Archetti-Horsfall formula (4): percent relatedness = $100 \times r_1 \times r_2 = \hat{R}$, say where r_1 = (heterologous response of strain 2)/(homologous response of strain 1) and r_2 = (heterologous response of strain 1)/(homologous response of strain 2). The level of significance of the *t* test was based on $\hat{r} = 2 \log_2 (\hat{R}/100)$ and the corresponding standard deviation.
[c] Estimated from the sum of squares pooled among rats (see reference 2). The null hypothesis tested was that the true relatedness ($\hat{R}$) is 100% (i.e., $r = 0$).

TABLE 3. Protective efficacy of vaccinia virus-RSV recombinants against homologous and heterologous intranasal RSV challenge

Primary immunization[a] with:	No. of animals	Challenge virus strain[b]	Virus recovered at 4 days after challenge infection					
			Nasal turbinates			Lung homogenates		
			No. shed (%)	Mean (log_{10}) titer ± SEM[c]	Reduction in titer[d]	No. shed (%)	Mean (log_{10}) titer ± SEM	Reduction in titer
Vaccinia-βgal	8	A2	8 (100)	6.4 ± 0.1		8 (100)	6.6 ± 0.1	
	8	18537	8 (100)	5.6 ± 0.1		8 (100)	4.8 ± 0.1	
Vaccinia-A2-F	9	A2	9 (100)	3.9 ± 0.3[e]	2.5	0 (0)[f]	<2.0[e]	≥4.6
	10	18537	9 (90)	3.2 ± 0.2[e]	2.4	1 (10)[f]	2.1 ± 0.1[e]	≥2.7[g]
Vaccinia-A2-G	11	A2	11 (100)	5.9 ± 0.1[h]	0.5	9 (82)	3.6 ± 0.3[e]	3.0[i]
	9	18537	9 (100)	4.6 ± 0.2[e]	1.0	7 (78)	2.9 ± 0.2[e]	1.9[i]

[a] Dose was 10^8 PFU given intradermally.
[b] Dose was $10^{5.4}$ PFU of the indicated RSV strain given intranasally at 21 days after primary immunization.
[c] Geometric mean (PFU/g). Minimum level of detectability was 10^2 PFU/g. Animals without detectable virus were assigned a titer of 2.0 for purposes of calculation.
[d] Compared with values for the corresponding vaccinia-βgal group.
[e] $P < 0.001$ by the two-tailed Student t test; compared with values for the corresponding vaccinia-βgal group.
[f] $P < 0.001$ by the two-tailed Fisher exact test; compared with values for the corresponding vaccinia-βgal group.
[g] Since only 1 of 10 animals had virus in the lungs, the absolute amount of reduction in replication could not be reliably assigned.
[h] $P < 0.01$ by the two-tailed Student t test; compared with values for the corresponding vaccinia-βgal group.
[i] Comparison of designated means: $t = 2.12$, df = 32, and $P < 0.05$; t calculated by using a test for the interaction of means.

titers for heterologous virus. This level of difference clearly discriminated between the subgroups in the relatedness calculations (Table 2) and confirmed previous work using a similar type of relatedness analysis (7, 17).

Protective efficacy of vaccinia virus-RSV recombinants against cross-subgroup challenge. The high level of cross-subgroup-reactive antibodies induced by the vaccinia virus-RSV A2 recombinants suggested that one or both of the recombinants might afford resistance to cross-subgroup RSV challenge infection. Cotton rats were immunized with either of the vaccinia virus-RSV recombinants and then were challenged intranasally with RSV subgroup A or B 21 days later. Animals previously immunized with vaccinia-βgal supported high levels of RSV replication in the nose and lungs (Table 3). Cotton rats previously immunized with vaccinia-A2-F demonstrated nearly complete resistance to lung infection by subgroup A (strain A2) or B (strain 18537) virus. Each of these animals had virus recovered from nasal turbinates, but the titers of both RSV subgroups were significantly reduced (250- to 300-fold) when compared with the titers of controls (Table 3). Importantly, the magnitude of reduction of the subgroup A and B viruses in nasal turbinates was comparable. The lack of complete protection in the noses of animals immunized with vaccinia-A2-F (or vaccinia-A2-G; see below) was most likely due to the failure of intradermally administered vaccinia virus to stimulate a local immune response in the upper respiratory tract (19).

Immunization with vaccinia-A2-G did not induce a level of protection against the heterologous virus comparable to that observed for the homologous virus. Approximately 80% of the animals had detectable virus in lung homogenates after challenge with RSV from either subgroup (Table 3). However, the level of replication in the lungs was significantly reduced (nearly 100- to 1,000-fold) for both subgroups when compared with controls. The reduction in homologous virus replication was approximately 13-fold more than the reduction in heterologous virus replication ($P < 0.05$; Table 3). As with vaccinia-A2-F, all of the challenged animals yielded virus from their noses. Although the levels of replication were significantly reduced for both strains (Table 3), the relatively low levels of reduction (compared with vaccinia-A2-F) did not permit separation of the effects with homologous and heterologous viruses.

DISCUSSION

In the present study, we measured the degree of antigenic relatedness between RSV subgroups A and B by analyzing polyclonal antibody responses induced by respiratory tract infection with RSV. Glycoprotein-specific assays of antibody responses induced by RSV infection of the respiratory tracts of cotton rats demonstrated that the F glycoproteins of the two RSV subgroups were highly related, whereas the G glycoproteins were approximately 20-fold different (~5% related; Table 2).

These levels of antigenic relatedness between the surface glycoproteins of the RSV subgroups were reflected in the protective efficacy of the vaccinia virus-RSV A2 recombinants. Cotton rats immunized with vaccinia-A2-F resisted infection of the lower respiratory tract when they were challenged by RSV of either subgroup (Table 3). Also, the partial protection of the upper respiratory tract conferred by vaccinia-A2-F was equally effective against both strains. Vaccinia-A2-G, while not conferring the complete protection observed with vaccinia-A2-F, did significantly reduce the amount of virus recovered from the lungs of rats challenged with virus from either subgroup. In contrast to animals immunized with vaccinia-A2-F, suppression of replication of the homologous virus was significantly greater (13-fold) in vaccinia-A2-G-immunized animals than that observed for the heterologous virus challenge virus. The difference directly reflected the 20-fold difference in the degree of antigenic relatedness between the G glycoproteins of subgroups A and B (Table 2).

These data provide a basis for preliminary recommendations concerning the antigenic composition for a subunit or recombinant DNA vaccine for RSV. Epitopes on the F glycoprotein responsible for inducing protective immune responses appear to be highly conserved between the RSV subgroups, which possibly accounts for the observed cross-subgroup protection in cotton rats. Also, in a finding consistent with previous results (16), the F glycoprotein appeared to play the major role in inducing host immunity. In contrast, the G glycoprotein induced a far less protective response to challenge by homologous virus and provided less complete cross-subgroup protection. Thus, the F glycoprotein appeared to play the predominant role in immunity to

challenge by both homologous and heterologous viruses. Therefore, it appears that an F glycoprotein from either subgroup, presented in a form that stimulates high levels of biologically active antibodies, might constitute the minimum requirement for a subunit vaccine.

The extant data are derived from experiments in which the challenge infection occurred less than a month after primary immunization. It is possible that the efficiency of protection from infection by heterologous virus wanes with time. Long-term challenge studies in animals as well as examinations of sequential isolates of repeat infections from humans will be necessary to assess the duration of protection from homologous and heterologous viruses. If resistance to heterologous virus is not as durable as resistance to homologous virus, additional subgroup-specific or cross-reactive antigens may be required for inclusion in an RSV vaccine.

The challenge to develop an RSV vaccine is considerable. In humans, naturally acquired immunity does not uniformly ameliorate illness until the third infection by RSV (12). Even then, immunity is incomplete, but it is sufficient to prevent serious illness. Furthermore, any strategy must overcome the effects of maternal antibodies and immunological immaturity on vaccine responses. Therefore, a reasonable goal for an RSV vaccine strategy is to induce sufficient protection to reduce the morbidity and mortality associated with the first and second infections. Whatever the strategy for immunoprophylaxis, the data presented in this study may help guide future decisions regarding the composition and testing of new RSV vaccines.

ACKNOWLEDGMENTS

We thank Robert M. Chanock, in whose laboratory this work was performed, for critical review of the manuscript; Ena Camargo and Robert L. Horswood for technical assistance; R. Michael Hendry for relating unpublished data and for monoclonal antibody strain typing; Praxis Biologics for the Long strain purified G glycoprotein; and Debra Brunelle and Linda Jordan for editorial assistance.

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