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Respiratory Syncytial Virus (RSV) F, G, M2 (22K), and N Proteins Each Induce Resistance to RSV Challenge, but Resistance Induced by M2 and N Proteins Is Relatively Short-Lived

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The ability of recombinant vaccinia viruses that separately encoded 9 of the 10 known respiratory syncytial virus (RSV) proteins to induce resistance to RSV challenge was studied in BALB/c mice. Resistance was examined at two intervals following vaccination to examine early (day 9) as well as late (day 28) immunity. BALB/c mice were inoculated simultaneously by the intranasal and intraperitoneal routes with a recombinant vaccinia virus encoding one of the following RSV proteins: F, G, N, P, SH, M, 1B, 1C, or M2 (22K). A parainfluenza virus type 3 HN protein recombinant (Vac-HN) served as a negative control. One half of the mice were challenged with RSV intranasally on day 9, and the remaining animals were challenged on day 28 postvaccination. Mice previously immunized by infection with RSV, Vac-F, or Vac-G were completely or almost completely resistant to RSV challenge on both days. In contrast, immunization with Vac-HN, -P, -SH, -M, -1B, or -1C did not induce detectable resistance to RSV challenge. Mice previously infected with Vac-M2 or Vac-N exhibited significant but not complete resistance on day 9. However, in both cases resistance had largely waned by day 28 and was detectable only in mice immunized with Vac-M2. These results demonstrate that F and G proteins expressed by recombinant vaccinia viruses are the most effective RSV protective antigens. This study also suggests that RSV vaccines need only contain the F and G glycoproteins, because the immunity conferred by the other proteins is less effective and appears to wane rapidly with time.

Infection with human respiratory syncytial virus (RSV), a paramyxovirus that is a major cause of bronchiolitis and pneumonia in infants and children, stimulates both cellular and humoral immune responses. It was recently shown that the two transmembrane surface glycoproteins, F and G, induce a neutralizing antibody response and stimulate resistance to RSV infection in cotton rats and mice (5, 11, 19, 21, 23). In addition, there is evidence that a number of RSV proteins may be recognized by class I major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTLs). These include the nucleocapsid-associated protein (N), the nonglycosylated, membrane-associated protein (variably designated 22K or M2), and the fusion or F glycoprotein (2, 10, 13, 14). However, the relative importance of these responses in resistance to RSV challenge following immunization remains to be fully characterized. Preliminary data indicate that immunization with vaccinia virus recombinants expressing the F or G glycoprotein (i.e., Vac-F or Vac-G) provides a high level of resistance to RSV challenge and that Vac-N induces only a low level of resistance (5, 7, 11, 19, 23).

In the present study, a series of nine vaccinia virus-RSV recombinants that each expressed a single RSV protein of the A2 strain (subgroup A) were evaluated for their abilities to induce resistance to RSV challenge in BALB/c mice. In this manner, each of the RSV proteins, except for the L polymerase protein, was studied separately for its potential role in immunity to RSV infection. The Vac-RSV recombinants were administered by both the intranasal (i.n.) and intraperitoneal (i.p.) routes to stimulate local and systemic mediators of immunity and thereby increase the possibility of detecting a protective effect of immunization. Serum neutralizing, radioimmune precipitation assay (RIPA) and F- or G-specific enzyme-linked immunosorbent assay (ELISA) antibodies were measured on day 0 and the day of challenge (day 9 or 28). Resistance to RSV infection was examined at two intervals following vaccination. This was done because BALB/c mice infected with RSV or Sendai virus attain peak CTL activity on days 7 to 10, whereas the humoral response is maximal after day 28 following i.n. infection (1, 6). In the current study, resistance to infection was examined early (day 9), at a time when direct CTL activity, i.e., CTL activity that does not require restimulation in vitro for its detection, was at its peak and the humoral response was building. Resistance was also examined late (day 28), at a time when the humoral response was maximal but when CTL activity would likely require restimulation of memory T cells (1). In this manner, the relative contributions of both early and late mediators of immunity could be assessed.

The Long strain (subgroup A) of RSV was used for infection and for assay of neutralizing antibodies. The virus was grown and titrated in HEp-2 cell monolayer cultures as previously described (17). Recombinant vaccinia viruses were grown and titrated in HEp-2 cells. The infectivity titer (PFU/ml) of the vaccinia virus recombinants was determined as previously described (2) by using an overlay containing 0.8 g of methylcellulose per 100 ml in Eagle's minimal essential medium with 2% fetal bovine serum. The production and characterization of recombinant vaccinia viruses expressing the RSV, F, G, N, P, M2, SH, or M or parainfluenza virus type 3 HN were as previously described (5, 10, 11, 18). Vaccinia viruses expressing 1B and 1C were produced and characterized in a similar fashion.

Six- to eight-week-old female BALB/c mice raised under specific pathogen-free conditions were obtained from the

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TABLE 1. Responses of mice immunized with vaccinia recombinant viruses or infected with RSV

Vaccination[a] (no. of animals)	Antibody titer (reciprocal mean $\log_2$ ± SE)[b] postinfection						RIPA antibody to homologous RSV protein (day 28)
	Neutralizing		ELISA-F		ELISA-G		
	Day 9	Day 28	Day 9	Day 28	Day 9	Day 28	
RSV (9)	6.8 ± 1.0	8.1 ± 1.5	11.1 ± 1.1	12.6 ± 1.0	4.5 ± 2.1	8.4 ±1.8	Positive[c]
Vac-F (9)	6.6 ± 1.1	8.5 ± 2.7	11.3 ± 1.3	14.3 ± 2.2	NT	4.0 ± 1.0	Positive
Vac-G (9)	4.2 ± 1.3	6.9 ± 2.9	NT	3.7 ± 0.8	4.7 ± 1.6	13.5 ± 2.5	Positive
Vac-M2 (22K) (10)	3.4 ± 1.1	≤3.3 ± 0	NT	3.5 ± 0.6	NT	3.5 ± 0.6	Negative[d]
Vac-N (10)	≤3.3 ± 0	≤3.3 ± 0	NT	3.5 ± 0.7	NT	≤3.3 ± 0	Positive
Vac-P (10)	≤3.3 ± 0	≤3.3 ± 0	NT	NT	NT	NT	Positive
Vac-SH (10)	3.6 ± 1.0	≤3.3 ± 0	NT	NT	NT	NT	Negative
Vac-M (10)	≤3.3 ± 0	≤3.3 ± 0	NT	NT	NT	NT	Negative
Vac-1B (10)	3.5 ± 0.6	≤3.3 ± 0	NT	NT	NT	NT	Negative
Vac-1C (10)	≤3.3 ± 0	≤3.3 ± 0	NT	NT	NT	NT	Negative
Vac-HN (9)	≤3.3 ± 0	3.4 ± 0.2	NT	3.7 ± 0.8	NT	3.9 ± 1.0	Negative

[a] Animals received a recombinant vaccinia virus (10^6 PFU) i.n. and i.p. or RSV (10^6) i.n. on day 0.

[b] The first dilution of the ELISAs was 1:40. Serum specimens without activity at this dilution were assigned a titer equivalent to one dilution lower (1:10). The first dilution of the plaque neutralization assay was 1:20, and specimens with no activity at this dilution were assigned a titer of 1:10. Day 0 ELISAs were performed on a sample of 20 animals from the experimental group. Day 0 titers for neutralizing, ELISA-F, and ELISA-G antibodies were ≤3.3 ± 0.0, 3.5 ± 0.6, and 3.7 ± 0.0, respectively. Each of the mice had an ELISA antibody response to the vaccinia virus. NT, No titer determined.

[c] Animals infected with RSV developed antibodies to the F, G, N, and P proteins.

[d] Negative for reaction with RSV proteins. Homologous reaction with the parainfluenza virus type 3 HN protein was not tested.

Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, Md.). Immunization, challenge, and bleeding from the retro-orbital venous plexus were performed by using methoxyflurane anesthesia. Animals were immunized with recombinant vaccinia viruses on day 0 with $10^{6.0}$ PFU administered both i.n. and i.p. in a 0.1-ml inoculum. A separate group of animals was infected i.n. with RSV (10^6 PFU/0.05 ml) on day 0. Half of the animals were challenged on day 9 and the remaining half were challenged on day 28. Four days following viral challenge, animals were sacrificed and lungs were harvested for virus titration as previously described (9, 17). Neutralizing antibodies were measured by a complement-enhanced, 60% plaque reduction neutralization assay (16). F- and G-specific antibodies were measured by ELISA (ELISA-F and -G antibodies, respectively) as previously described (16). RIPAs of sera were carried out by using [^{3}H]glucosamine, [^{35}S]methionine, or [^{35}S]methionine plus [^{35}S]cysteine-labeled, RSV-infected HEp-2 cell lysates as previously described (12), except that the samples were analyzed by sodium dodecyl sulfate (SDS)–17% polyacrylamide gel electrophoresis.

The neutralizing, ELISA-F, ELISA-G, and RIPA antibody responses are presented in Table 1. Immunization with Vac-F induced a high titer of ELISA-F antibodies. There was an eightfold rise in mean titer from day 9 to day 28 comparable with the increment attained by mice infected with RSV. Each of the mice immunized with Vac-G also developed a high titer of ELISA-G antibodies on day 28, which greatly exceeded that achieved following RSV infection. Vac-F-immunized animals developed a titer of neutralizing antibodies on days 9 and 28 comparable with that of RSV-infected animals. Mice immunized with Vac-G developed a neutralizing antibody titer lower than that of Vac-F-immunized or RSV-infected animals. Animals immunized with Vac-M2, -N, -P, -SH, -M, -1B, -1C, or -HN did not develop a significant neutralizing antibody response, but radioimmunoprecipitation antibodies (RIPA antibodies) were induced by Vac-N or Vac-P.

Immunization with Vac-F or infection with RSV induced complete pulmonary resistance to RSV challenge on day 9 or day 28 (Table 2). Mice immunized with Vac-G were completely resistant to challenge on day 9 and almost completely resistant on day 28 ($P < 0.001$). Animals immunized with Vac-M2 were highly resistant to challenge on day 9. Resistance was also evident following challenge on day 28, but there was a significant diminution in resistance compared with that at day 9. Animals previously infected with Vac-N showed a low but significant level of resistance on day 9. This effect was transient, because Vac-N-immunized mice did not exhibit resistance on day 28. Animals which received Vac-P, -SH, -M, -1B, -1C, or -HN were not resistant to replication of RSV on either day 9 or day 28.

Previous studies identified the F and G glycoproteins as the major mediators of resistance to infection with RSV (5, 11, 19–23). In addition, the N protein was also observed to provide partial protection (7). The present study extends these previous studies in two ways. First, an immune response to an M2 protein was associated with a high level of resistance in BALB/c mice, and the ability of the N protein to induce a low but significant level of resistance was confirmed. Importantly, the other RSV proteins (SH, M, P, 1B, and 1C) failed to induce resistance under the experimental conditions used. Second, the resistance induced by the M2 gene product or the N protein rapidly waned after day 9 to a low or undetectable level by day 28. This was in contrast to the resistance induced by the F or G glycoprotein which appeared early (day 9) and was maintained at a high level on day 28. These observations can be interpreted in the context of what is known about the immune response of BALB/c mice to RSV.

First, the F and G glycoproteins induce neutralizing antibodies which are known to be protective against RSV challenge (5, 11, 19, 21, 23). The F, but not the G, glycoprotein is also a known target for RSV-specific CTLs (2, 10, 14). Thus, the high level of resistance induced by the Vac-F immunization may have been mediated in part by MHC class I-restricted CTLs. However, it is clear that a high level of resistance can be induced by a RSV protein, such as the G glycoprotein, that is not known to be a target of CTLs. This is not surprising, because cotton rats with a passively acquired RSV serum neutralizing antibody titer of approxi-

TABLE 2. Immunization of mice with certain vaccinia virus-RSV recombinants induces resistance to RSV challenge[a]

Animals immunized with:	Day 9				Day 28			
	No. of mice tested	No. of mice in which virus was detected	Virus titer[b]	Log_{10} fold reduction in titer[c]	No. of mice tested	No. of mice in which virus was detected	Virus titer[b]	Log_{10} fold reduction in titer[c]
RSV	10	0	$<1.7 \pm 0.0$[d]	≥ 2.8	10	0	$<1.7 \pm 0.0$[d]	≥ 2.9
Vac-F	10	1	$<1.7 \pm 0.1$[d]	≥ 2.8	10	0	$<1.7 \pm 0.0$[d]	≥ 2.9
Vac-G	10	0	$<1.7 \pm 0.0$[d]	≥ 2.8	10	2	2.0 ± 0.6[d]	2.6
Vac-M2	10	4	2.0 ± 0.4[d]	2.5	10	10	3.7 ± 0.5[d]	0.9
Vac-N	10	9	3.7 ± 0.8[d]	0.8	10	10	4.4 ± 0.3	0.2
Vac-P	10	10	4.0 ± 0.2	0.5	9	9	4.2 ± 0.7	0.4
Vac-SH	8	8	4.3 ± 0.3	0.2	10	10	4.8 ± 0.4	0
Vac-M	10	10	4.0 ± 0.3	0.5	9	9	4.7 ± 0.5	0
Vac-1B	9	9	4.1 ± 0.5	0.4	10	10	4.8 ± 0.6	0
Vac-1C	10	10	4.5 ± 0.4	0	10	10	4.8 ± 0.5	0
Vac-HN	10	10	4.5 ± 0.4		9	9	4.6 ± 0.6	

[a] Animals received a recombinant vaccinia virus (10^6 PFU) i.n. and i.p. or RSV (10^6) i.n. on day 0. Mice were challenged i.n. with 10^6 PFU/0.05 ml of RSV on day 9 or 28, and lungs were removed 4 days later for quantitation of virus.

[b] Mean log_{10} titer ± standard error (PFU/gram of tissue). The lowest level of virus detectable in this system was 1.7 PFU/g; lung homogenates lacking detectable virus were assigned a titer of $10^{1.7}$ PFU/g.

[c] Reduction of replication was calculated by subtracting the mean log_{10} titer of RSV- or Vac-RSV-infected animals from the corresponding titer of control mice infected with Vac-HN.

[d] Significant protection at $P < 0.001$ in an independent t test. The statistical significance of diminished protection from day 9 to 28 was determined by using a pooled measure of variability of the Vac-22K, -N, and -HN groups.

mately 1:300 are completely resistant to pulmonary replication of RSV (15). Second, the resistance induced by the M2 protein or N protein is likely mediated by a cellular immune response, probably a MHC class I-restricted CTL response. A neutralizing or RIPA antibody response to the M2 protein was not detected, which suggests that humoral immunity did not play a role in the resistance induced by Vac-M2. The N protein induced RIPA antibodies that lacked neutralizing activity. Since the N protein is not expressed on the surface of infected cells, it is unlikely that this nonneutralizing antibody contributed significantly to the resistance observed. This suggestion is offered with the caveat that nonneutralizing antibodies have been associated with resistance to some viruses. The suggestion that the resistance induced by the M2 protein or the N protein is mediated by a MHC class I-restricted CTL response is consistent with the observations that these gene products have been shown to be CTL target antigens in BALB/c mice (2, 10, 13). Conversely, the M, P, SH, and 1B proteins are not known CTL target antigens, and vaccinia virus recombinants bearing genes for these proteins failed to induce resistance. It is important to note that the M2 gene has two open reading frames (4) that are conserved in both subgroups of RSV (3), and thus this gene might encode an 11th RSV protein which remains to be identified. The Vac-M2 recombinant used in this study contained both open reading frames of the M2 gene; therefore, products of either or both open reading frames could be responsible for the protective immunity observed. The resistance induced by the M2 or N protein was highest early (day 9) rather than later (day 28) after infection. This observation is consistent with the time course of activity of the MHC class I-restricted CTL response, which peaks early (day 3 to 7) during a paramyxovirus infection and is not detectable after day 14 (1, 6). The findings from the present study would suggest that direct CTLs are more effective in restricting replication of challenge RSV than memory CTLs. Studies are in progress to further characterize the immunological mediators of resistance induced by the N and M2 gene products.

These observations have implications for development of vaccines for viruses such as RSV which cause an acute illness that evolves rapidly after infection. Vaccines against such viruses that rely solely on a MHC class I-restricted CTL response would likely provide only transient resistance to infection. This suggestion is consistent with the epidemiological observation that repeated past infections with influenza A viruses containing shared MHC class I-restricted CTL epitopes provide little resistance to illness caused by new antigenic variants. These new variants possess CTL epitopes shared with prior influenza A virus strains but contain variant hemagglutinins and are poorly neutralized by antibodies induced by infection with the previously circulating strains (8). Prolonged resistance to infection with RSV is more likely to be induced by a vaccine which produces a protective humoral immune response. The major antigens to be included in an RSV vaccine are the F and G glycoproteins, which efficiently stimulate neutralizing antibodies.

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