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Resistance to Respiratory Syncytial Virus (RSV) Challenge Induced by Infection with a Vaccinia Virus Recombinant Expressing the RSV M2 Protein (Vac-M2) Is Mediated by CD8$^+$ T Cells, While That Induced by Vac-F or Vac-G Recombinants Is Mediated by Antibodies

MARK CONNORS,[1]* ARUN B. KULKARNI,[1] PETER L. COLLINS,[1] CAI-YEN FIRESTONE,[1] KEVIN L. HOLMES,[2] HERBERT C. MORSE III,[2] AND BRIAN R. MURPHY[1]

Respiratory Viruses Section, Laboratory of Infectious Diseases,[1] and Laboratory of Immunopathology,[2] National Institute of Allergy and Infectious Diseases, Building 7, Room 106, 9000 Rockville Pike, Bethesda Maryland 20892

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It was previously demonstrated that the vaccinia virus recombinants expressing the respiratory syncytial virus (RSV) F, G, or M2 (also designated as 22K) protein (Vac-F, Vac-G, or Vac-M2, respectively) induced almost complete resistance to RSV challenge in BALB/c mice. In the present study, we sought to identify the humoral and/or cellular mediators of this resistance. Mice were immunized by infection with a single recombinant vaccinia virus and were subsequently given a monoclonal antibody directed against CD4$^+$ or CD8$^+$ T cells or gamma interferon (IFN-γ) to cause depletion of effector T cells or IFN-γ, respectively, at the time of RSV challenge (10 days after immunization). Mice immunized with Vac-F or Vac-G were completely or almost completely resistant to RSV challenge after depletion of both CD4$^+$ and CD8$^+$ T cells prior to challenge, indicating that these cells were not required at the time of virus challenge for expression of resistance to RSV infection induced by the recombinants. In contrast, the high level of protection of mice immunized with Vac-M2 was completely abrogated by depletion of CD8$^+$ T cells, whereas depletion of CD4$^+$ T cells or IFN-γ resulted in intermediate levels of resistance. These results demonstrate that antibodies are sufficient to mediate the resistance to RSV induced by the F and G proteins, whereas the resistance induced by the M2 protein is mediated primarily by CD8$^+$ T cells, with CD4$^+$ T cells and IFN-γ also contributing to resistance.

Respiratory syncytial virus (RSV), a member of the *Pneumovirus* subgroup of the *Paramyxoviridae* family, is the major cause of bronchiolitis and viral pneumonia in infants and children. Development of a safe, effective vaccine would be facilitated by an understanding of the contributions of individual viral proteins to resistance to infection as well as the immune mechanisms which mediate this resistance. Recently, immunization of animals with vaccinia virus recombinants each encoding a single RSV protein has been used to study the contribution of individual RSV proteins to resistance to RSV challenge as well as to study the humoral and cellular immune responses that are induced. Previous studies have demonstrated that the vaccinia virus recombinants expressing the RSV F, G, or M2 protein (Vac-F, Vac-G, or Vac-M2, respectively) each induced a high level of resistance to RSV challenge in BALB/c mice (9, 23, 30, 33). However, the relative contributions of the humoral and cellular arms of the immune response to resistance induced by the vaccinia virus-RSV recombinants remained unknown. Since the F glycoprotein induces neutralizing antibodies (9, 24, 33) and is a target for major histocompatibility complex class I-restricted CD8$^+$ cytotoxic T cells (CTLs) (22, 26), it is possible that the resistance induced by Vac-F is mediated by T cells, antibodies, or both. In contrast, the M2 protein is a poor inducer of serum antibodies but is the major target antigen of CTLs in BALB/c mice (22, 23, 25). Previous studies have demonstrated that passive transfer of RSV-specific CTLs can clear RSV from the lungs of infected mice (7, 8, 19). Hence, the resistance induced by Vac-M2 could be due primarily to the cellular arm of the immune response. Since both CD8$^+$ and CD4$^+$ T cells can have antiviral activity in vivo (4, 6, 17, 28, 31), it was necessary to examine the contribution of each of these T-cell subsets to the immunity induced by vaccinia virus-RSV recombinants. Since gamma interferon (IFN-γ) has been implicated as a contributor to the antiviral properties of T cells, its contribution to the resistance induced by vaccinia virus-RSV recombinants was also assessed. In the present studies, BALB/c mice were infected with Vac-F, Vac-G, or Vac-M2, and just prior to RSV challenge (10 days after immunization), treatment (on days 6, 9, and 12 after immunization) with monoclonal antibodies specific for CD4$^+$ or CD8$^+$ T cells or IFN-γ was initiated. CD4$^+$ or CD8$^+$ T cells or IFN-γ induced by immunization would be depleted at the time of challenge by the monoclonal antibody treatment, whereas RSV-specific antibodies would be expected to be unaffected. The levels of RSV replication in normal mice and T-cell- or IFN-γ-depleted mice were then compared.

The Long strain (a member of the A subgroup) of RSV was used throughout. The virus was grown in HEp-2 cells and titrated for infectivity by plaque formation in HEp-2 cell monolayer cultures as previously described (20, 27). RSV was purified from cell culture supernatants on an RK continuous flow centrifuge with a J1 rotor at 40,000 rpm over a continuous sucrose gradient containing 100 mM magnesium sulfate, 50 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, and 150 mM sodium chloride.

* Corresponding author.

Recombinant vaccinia viruses were grown and titrated in HEp-2 cells as previously described (3, 9). The production and characterization of recombinant vaccinia viruses expressing the RSV A2 strain (also a member of the RSV A subgroup) F, G, M2, or parainfluenza virus type 3 hemagglutinin-neuraminidase protein (Vac-HN) were previously described (10, 22, 24, 29).

Monoclonal antibodies were prepared as ascitic fluids of hybridoma-inoculated pristane-primed *nu/nu* mice by Bioproducts for Science (Indianapolis, Ind.). Antibodies were partially purified by precipitation with 50% ammonium sulfate and dialyzed against phosphate-buffered saline to a final concentration of 1 mg of immunoglobulin G (IgG) as determined by anti-rat IgG immunodiffusion plates (ICN Pharmaceuticals Inc., Costa Mesa, Calif.).

Six- to eight-week-old female BALB/c mice that had been raised under specific pathogen-free conditions were obtained from the Charles River Laboratories (Cambridge, Mass.). We performed immunization, challenge, and bleeding from the retroorbital venous plexus of mice under methoxyflurane anesthesia. Animals were immunized with recombinant vaccinia viruses on day 0 with 10^6 PFU administered intranasally (i.n.) and 10^6 PFU administered intraperitoneally, each in a 0.05-ml inoculum. A separate group of animals was infected i.n. with RSV (10^6 PFU/0.05 ml) on day 0. A brief description of the monoclonal antibody preparations used, their schedule of administration, and the determination of depletion at the time of RSV challenge by fluorescence-activated cell sorter (FACS) (FACStar Plus; Becton-Dickinson, Mountain View, Calif.) is given in footnote *a* of Table 1 and footnote *b* of Table 2. On day 10, mice were bled and then challenged with 10^6 PFU of RSV i.n. On day 14, animals were sacrificed, and lungs were harvested and titrated for virus as previously described (20, 27).

The results of FACS analysis of spleen cell suspensions from mice depleted of $CD4^+$, $CD8^+$, or both $CD4^+$ and $CD8^+$ T cells or IFN-γ are shown in Table 1. The fluorescein isothiocyanate (FITC)-labelled antibody against rat IgG, which was used to control for viable cells that may have had their antigenic sites masked by the rat monoclonal antibody administered in vivo, stained <2% of the spleen cells. This indicated that the low frequencies of $CD4^+$ or $CD8^+$ T cells in mice depleted of these cells, as detected by FITC-labelled antibody added in vitro for FACS analysis, were not artifacts of blocking by the rat monoclonal antibody administered in vivo. Mice treated with anti-CD4 monoclonal antibody were effectively depleted of $CD4^+$ T cells. The frequency of cells staining with Thy-1.2, which binds both CD4 and CD8 subsets of T cells, closely approximated the sum of $CD8^+$, $CD4^+$, and anti-rat IgG^+ cells, indicating that the $CD4^+$ cells detected with FITC-labelled anti-CD4 antibody accurately reflected the frequency of viable helper T cells in the spleen cell population. Mice treated with anti-CD8 monoclonal antibody were effectively depleted of $CD8^+$ cells. Animals receiving anti-CD4 and anti-CD8 antibodies were effectively depleted of both T-cell subsets. Mice which received XMG-6 (anti-IFN-γ), which is not expected to affect the frequencies of $CD4^+$ or $CD8^+$ T cells, had levels of T-cell subsets similar to those of mice receiving the control monoclonal antibody preparation.

Mice immunized with Vac-G or Vac-F maintained a high level of resistance to RSV challenge despite depletion of T cells or IFN-γ (Table 2). In contrast, the resistance induced by immunization with Vac-M2 was totally abrogated by depletion of $CD8^+$ T cells. Mice immunized with Vac-M2 and depleted of $CD4^+$ T cells or IFN-γ had intermediate levels of resistance. Although the level of RSV replication in mice immunized with Vac-M2 and depleted of $CD4^+$ T cells or IFN-γ was not significantly different ($P > 0.05$) from that in Vac-M2-immunized nondepleted mice, the frequency of animals with detectable virus was significantly increased ($P < 0.02$).

TABLE 1. Treatment of mice with monoclonal antibodies to $CD4^+$ or $CD8^+$ T-cell subsets effectively depletes these T cells from mouse spleens

MAb treatment of mice[a]	Antigen recognized by MAb	Frequency (%) of indicated antigen-positive cells[b]				
		Thy-1.2$^+$	CD8$^+$	CD4$^+$	FITC-Av	rIg$^+$
None	None	19.8	7.2	14.4	0.2	0.2
SFR3D5	Control	29.0	10.9	21.9	0.2	0.1
GK 1.5	CD4	18.9	15.2	0.3	0.2	0.2
2.43	CD8	20.6	0.2	26.0	0.2	0.1
GK 1.5 + 2.43	CD4, CD8	3.2	0.1	0.3	0.1	0.1
XMG-6	IFN-γ	23.1	8.7	16.9	0.2	0.1

[a] On days 6 and 9, mice received 1 mg of a single monoclonal antibody (MAb): GK 1.5 (to deplete $CD4^+$ cells), 2.43 (to deplete $CD8^+$ cells), GK 1.5 and 2.43, or SFR3D5 (a rat IgG directed at a human leukocyte antigen which served as a control). On day 9 only, a separate group of mice received 1 mg of XMG-6, which was used to deplete the soluble protein IFN-γ and is not expected to alter T-cell subsets. Ten days after intranasal RSV infection (10^6 PFU/0.05 ml of inoculum), a single mouse from each monoclonal antibody treatment group or an untreated mouse was sacrificed to determine the extent of depletion of the appropriate T-cell subset(s).

[b] The numbers are the percentage of spleen cells from individual mice reactive with FITC-labelled antibodies to Thy-1.2, CD8, rat immunoglobulin (rIg), or biotin-labelled antibody to CD4 complexed with FITC-avidin (FITC-Av) conjugate as detected by FACS. The frequency of cells binding FITC-Av alone was included to control the nonspecific FITC-Av binding. The rIg antibody was used to detect viable cells which may have the passively transferred monoclonal antibody blocking the antigenic site of the FITC-labelled antibody used in the assay. The rIg antibody was used at a saturating concentration that was previously determined with unlabelled GK 1.5 antibody bound to normal mouse spleen cells.

The results of the present study confirm and extend previous observations of the mechanism of protection induced by the RSV F, G, and M2 proteins in several ways. First, the protection induced by the F protein, which is known to stimulate serum-neutralizing antibodies and is a known target antigen for RSV-specific CTLs, was not affected by depletion of $CD4^+$ or $CD8^+$ T cells or IFN-γ. It should be noted that the high level of resistance conferred by antibodies to the F glycoprotein may have obscured detection of a component of resistance that may have been mediated by CTLs. Similarly, the protection induced by the G glycoprotein, which is known to stimulate serum-neutralizing antibodies and is not a known target antigen for CTLs, was not affected by depletion of either T-cell subset or IFN-γ. These data are consistent with observations in our laboratory in which passive immunization of cotton rats with both Vac-F- and Vac-G-immune serum is able to protect against RSV challenge. However, it is also possible that the resistance in Vac-F- or Vac-G-immunized animals depleted of $CD4^+$ or $CD8^+$ T cells observed was not mediated by serum antibodies alone. The vaccinia virus recombinant immunizations in the present study were administered both i.n. and intraperitoneally. It is therefore possible that Vac-F or Vac-G immunization induced mucosal IgA antibodies which mediated the observed resistance. Alternatively, and less likely, this resistance may have been mediated locally by residual low levels of $CD4^+$ or $CD8^+$ T cells or IFN-γ in depleted animals. It appears most likely on the basis of the

TABLE 2. Resistance against RSV challenge induced by Vac-M2, but not Vac-F or Vac-G, is ablated by depletion of $CD8^+$ T cells

Source of infection	Replication of RSV challenge virus in control or T-cell depleted mice previously infected with Vac-RSV recombinants or with RSV[a]									
	Nondepleted		CD4 depleted		CD8 depleted		CD4 + CD8 depleted		IFN-γ depleted	
	No. of mice with detectable virus/no. of mice tested	Virus titer ± SE[b]	No. of mice with detectable virus/no. of mice tested	Virus titer ± SE	No. of mice with detectable virus/no. of mice tested	Virus titer ± SE	No. of mice with detectable virus/no. of mice tested	Virus titer ± SE	No. of mice with detectable virus/no. of mice tested	Virus titer ± SE
Vac-F	0/4	≤1.6 ± 0.0[c]	1/4	1.8 ± 0.3[c]	0/3	≤1.6 ± 0.0[c]	0/4	≤1.6 ± 0.0[c]	1/4	1.9 ± 0.6[c]
Vac-G	6/9	2.4 ± 0.6[c]	4/10	2.2 ± 0.7[c]	2/10	1.8 ± 0.5[c]	2/10	1.8 ± 0.4[c]	5/10	2.3 ± 0.8[c]
Vac-M2	2/8	1.9 ± 0.6[c]	9/10[d]	3.0 ± 0.8	9/9[d]	4.8 ± 0.5[e]	9/8[d]	4.5 ± 0.9[e]	9/10[d]	3.3 ± 0.9
Vac-HN	9/9	4.8 ± 0.5	10/10	4.6 ± 0.9	9/9	4.7 ± 0.6	9/9	4.5 ± 0.3	9/9	4.8 ± 0.5
RSV	0/5	≤1.6 ± 0.0[c]	0/4	≤1.6 ± 0.0[c]	0/5	≤1.6 ± 0.0[c]	0/5	≤1.6 ± 0.0[c]	0/6	≤1.6 ± 0.0[c]

[a] BALB/c mice were infected on day 0 with RSV (10^6 PFU/0.05 ml of inoculum i.n. or a vaccinia recombinant encoding a single RSV protein (10^6 PFU/0.05 ml i.n. and intraperitoneally). BALB/c mice were challenged with RSV (10^6 PFU/0.05 ml) on day 10, and lungs were removed 4 days later for quantitation of virus. Mice received a control antibody, GK 1.5, 2.43, or GK 1.5 and 2.43 antibody preparations, as outlined in footnote *a* of Table 1, on days 6, 9, and 12 after immunization. XMG-6 (anti-IFN-γ) was administered on day 9 only.

[b] Mean $\log_{10}$ titer (plaque-forming units per gram of tissue) ± standard error (SE). The lowest level of virus detectable in this system was $10^{1.7}$ PFU/gm and lung homogenates lacking detectable virus were assigned a titer of $10^{1.6}$ PFU/ml.

[c] Significant protection at the 0.05 level in an independent *t*-test compared with that of Vac-HN-immunized animals which received the same antibody preparation.

[d] Significant increase in number of animals with detectable pulmonary virus at the 0.02 level in a Fisher's exact test compared with nondepleted Vac-M2-immunized mice.

[e] Significant increase in pulmonary virus titer at the 0.05 level in an independent *t*-test compared with that for nondepleted Vac-M2-immunized mice.

data in the present study that serum or local antibodies alone are sufficient for the resistance induced by the F or G glycoprotein.

In contrast, the protection induced by the M2 protein, which is a major CTL target antigen but does not induce detectable serum-neutralizing antibodies, was totally abrogated by depletion of $CD8^+$ T cells, indicating that these cells mediate the protection induced by the M2 protein. Specifically, Vac-M2-immunized mice depleted of $CD8^+$ T cells had pulmonary virus titers comparable to those of Vac-HN-immunized mice treated with control monoclonal antibody, indicating that $CD4^+$ T cells, present in numbers similar to those of control animals and presumably functioning normally, do not appear to directly mediate the resistance induced by this protein. Although $CD4^+$ T cells do not appear to directly mediate the protection induced by the M2 protein, Vac-M2-immunized mice depleted of $CD4^+$ T cells had pulmonary virus titers intermediate between those of mice depleted of $CD8^+$ T cells and those of mice which received a control preparation, indicating that $CD4^+$ T cells contribute to this protection most likely through providing T-cell "help." Although the role of $CD4^+$ T cells in the generation of CTLs is widely accepted, it has recently been demonstrated with a number of viral systems that primary as well as memory CTL responses can be generated in the absence of $CD4^+$ T cells (1, 13, 15, 18, 21). In the present study, it is possible that the intermediate level of protection observed in Vac-M2-immunized $CD4^+$ T-cell-depleted mice was mediated by a $CD4^+$ T-cell-independent subpopulation of $CD8^+$ T cells, $CD8^+$ T cells responding to the residual very low levels of $CD4^+$ T cells, or, alternatively, residual $CD8^+$ T cells generated prior to $CD4^+$ T-cell depletion. Regardless of the mechanism, it appears that $CD4^+$ T cells contribute to the protection induced by the M2 protein.

Vac-M2-immunized animals depleted of IFN-γ also had pulmonary virus titers that were intermediate between those of mice depleted of $CD8^+$ T cells and those of mice which received a control preparation. IFN-γ is produced by $CD4^+$ and $CD8^+$ T cells as well as cells which are not of T-cell lineage (e.g., natural killer cells [12] and in athymic nude mice [2]) and has protean biologic effects, including a prominant role as a lymphokine, direct antiviral activity, and enhancement of expression of major histocompatibility complex-encoded antigens (14, 32). Depletion of IFN-γ has been shown to diminish CTL activity as well as to impair clearance of a number of viruses (11, 16, 32). Although the mechanism of action of IFN-γ in mediating resistance is undefined, the present study demonstrates that it does contribute to the resistance induced by the M2 protein.

The results in Vac-M2-immunized mice indicate that $CD8^+$ T-cell effector mechanisms, most likely including direct killing of infected targets or cellular antiviral changes induced by production of IFN-γ or both, are required for resistance to RSV challenge. $CD4^+$ T cells may also contribute to resistance, but this effect cannot be detected, with the parameters measured in this study, in the absence of $CD8^+$ T cells. The mechanisms by which the $CD4^+$ T-cell subset may contribute to resistance could include production of interleukin-2 and facilitation of expansion of $CD8^+$ effectors, as well as production of IFN-γ. Although it is clear from the present study that Vac-M2 induces $CD8^+$ T cells which mediate protection against RSV infection, their importance in immunity to reinfection with RSV is uncertain. We have previously demonstrated that the resistance induced by immunization with Vac-M2 wanes with time and is significantly reduced by day 28 after infection. Given its short-lived nature, it is reasonable to suggest that this resistance is mediated by primary CTLs (i.e., those induced by the immunization and not requiring restimulation from the memory $CD8^+$ T-cell population) rather than CTLs restimulated from memory during RSV challenge. However, it is possible that M2 protein-specific CTLs restimulated from memory during challenge, although not affecting peak titers of virus achieved with this rapidly replicating virus, play a role by accelerating viral clearance from immune mice. Studies are in progress to further characterize the direct and memory CTL responses to Vac-M2 and their role in protection and viral clearance. Lastly, it should be emphasized that the

pattern of resistance induced by the RSV proteins in the present study are true only for BALB/c mice. Preliminary data in our laboratory indicate that the ability of individual vaccinia virus-RSV recombinants to induce resistance to RSV infection varies widely between mouse strains. It would be expected that the ability of individual RSV proteins to induce humoral or cellular mediators of immunity would vary between both mice and humans as well as within human populations.

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