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The Cytolytic Activity of Pulmonary CD8$^+$ Lymphocytes, Induced by Infection with a Vaccinia Virus Recombinant Expressing the M2 Protein of Respiratory Syncytial Virus (RSV), Correlates with Resistance to RSV Infection in Mice

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Previous studies demonstrated that the pulmonary resistance to respiratory syncytial virus (RSV) challenge induced by immunization with a recombinant vaccinia virus expressing the M2 protein of RSV (vac-M2) was significantly greater 9 days after immunization than at 28 days and was mediated predominantly by CD8$^+$ T cells. In this study, we have extended these findings and sought to determine whether the level of CD8$^+$ cytotoxic T-lymphocyte (CTL) activity measured in vitro correlates with the resistance to RSV challenge in vivo. Three lines of evidence documented an association between the presence of pulmonary CTL activity and resistance to RSV challenge. First, vac-M2 immunization induced pulmonary CD8$^+$ CTL activity and pulmonary resistance to RSV infection in BALB/c (*H-2^d*) mice, whereas significant levels of pulmonary CTL activity and resistance to RSV infection were not seen in BALB.K (*H-2^k*) or BALB.B (*H-2^b*) mice. Second, pulmonary CD8$^+$ CTL activity was not induced by infection with other vaccinia virus-RSV recombinants that did not induce resistance to RSV challenge. Third, the peak of pulmonary CTL activity correlated with the peak of resistance to RSV replication (day 6), with little resistance being observed 45 days after immunization. An accelerated clearance of virus was not observed when mice were challenged with RSV 45 days after immunization with vac-M2. The results indicate that resistance to RSV induced by immunization with vac-M2 is mainly mediated by primary pulmonary CTLs and that this resistance decreases to very low levels within 2 months following immunization. The implications for inclusion of CTL epitopes into RSV vaccines are discussed in the context of these observations.

Cytotoxic T lymphocytes (CTLs) play a major role in host defenses in most viral infections. CTLs control acute infection directly by destruction of cells producing virus (13) and possibly by the release of cytokines with antiviral activity such as gamma interferon (13). The generation of the CTL response is associated with clearance of virus-infected cells, suggesting a contribution to resolution of certain viral infections (1, 7, 24, 34, 40). Antiviral CD8$^+$ CTLs recognize short peptides derived from viral proteins when bound to major histocompatibility complex (MHC) class I molecules. In addition, MHC class II-restricted CD4$^+$ T cells are also essential for recovery from certain viral infections (29), and in some cases these cells kill infected cells through the recognition of viral peptides complexed with MHC class II antigens (21).

Respiratory syncytial virus (RSV), a member of the *Pneumovirus* genus of the *Paramyxoviridae* family, is the leading cause of severe lower respiratory tract infection in infants. An understanding of the contributions of individual viral proteins to resistance to infection as well as the immune mechanisms that mediate resistance is essential for the development of a safe and effective vaccine against RSV. Previous studies from our laboratory have demonstrated that F, G, and M2 (also designated 22K) proteins of RSV are protective antigens in BALB/c mice (9). However, the resistance induced by vaccinia virus recombinant vac-M2 differed from that of vac-F and vac-G in that the resistance induced by vac-M2 was very short-lived. Mice immunized with vac-M2 protein exhibited significant resistance on day 9, which largely waned by day 28 (9). It was also shown that resistance induced by immunization with vac-M2 was mediated by CD8$^+$ T cells (10). These observations are consistent with the finding that the M2 protein is a major target antigen for RSV-specific CTLs in BALB/c (*H-2^d*) mice (30, 32). In this study, we sought to extend our understanding of the immunity to RSV infection induced by immunization with vac-M2 to further characterize the duration of immunity offered by CD8$^+$ CTLs and to identify the in vitro correlates of this resistance.

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MATERIALS AND METHODS

Viruses. The Long strain (a member of the A subgroup) of RSV was used throughout except for the CTL assays, for which the A2 strain (also subgroup A) was used. The virus stocks were grown in HEp-2 cells and titrated for infectivity by plaque assay in HEp-2 cells as described elsewhere (27, 33). Recombinant vaccinia viruses were grown and titrated by plaque assay in HEp-2 monolayers, using Eagle's minimal essential medium (Quality Biologicals, Gaithersburg, Md.) supplemented with 5% fetal bovine serum (FBS). The production and characterization of recombinant vaccinia viruses expressing the RSV F, G, M, N, P, M2, 1B, 1C, or SH or parainfluenza virus type 3 HN protein has been described in detail elsewhere (12, 31, 35).

Mice and immunization. Six- to eight-week-old BALB/c (*H-2^d*) female mice were obtained from the Frederick Cancer Research and Development Center of the National Cancer

Institute (Frederick, Md.). Ten- to twelve-week-old BALB.B (H-2^b) and BALB.K (H-2^k) female mice were kindly provided by R. Schwartz, National Institute of Allergy and Infectious Diseases. Mice were anesthetized with methoxyflurane for immunization and challenge. Infection with RSV was performed by intranasal (i.n.) administration of 10^6 PFU in a 0.05-ml inoculum. Recombinant vaccinia viruses were administered both by i.n. and intraperitoneal (i.p.) routes (10^6 PFU/ 0.05 ml at each site).

Cells and antibodies. Target cells for CTL assays were either a BALB/c fibroblast line acutely infected with the A2 virus or BCH4 cells (kindly provided by Bruce Fernie), which are a BALB/c fibroblast cell line persistently infected with the Long strain of RSV (16). L929 cells (H-2^k), a fibroblast line from a C3H mouse, and YAC-1 cells (H-2^a), a line derived from a Moloney leukemia virus-induced lymphoma in an A/Sn mouse (22), were kindly provided by G. Karupiah. Hybridoma lines secreting anti-CD8 (3.115) or anti-CD4 (RL.172) antibodies were obtained from the American Type Culture Collection (Rockville, Md.). These lines were grown in Iscove's modified Dulbecco's medium (Quality Biologicals) supplemented with 5% FBS and 20 mM L-glutamine (IMDM+). The antibodies secreted by these lines were used as undiluted culture supernatant.

CTL assay. Primary in vivo effector cells were prepared from lungs of i.n.-infected mice as described previously (5). Cells were washed three times with Hanks balanced salt solution and resuspended in IMDM+ supplemented with 2-mercaptoethanol (5×10^{-4} M). Secondary in vitro effector cells were prepared from the splenocytes of the primed mice by restimulating the splenocytes with 0.5 PFU of RSV per cell for 5 days. For labeling, 10^6 BCH4 cells, BALB/c fibroblasts, or L929 cells were pelleted and incubated with 50 μl of $Na_2{}^{51}CrO_4$ (^{51}Cr) at a concentration of 10 mCi/ml for 1 h. Labeled cells were washed three times with IMDM+ and resuspended. When L929 cells were used as targets, cells were infected with 10 PFU of RSV per cell for 18 h prior to the assay.

For the CTL assay, effector cells were placed in triplicate into microtiter wells (100 μl) at appropriate dilutions, after which 10^4 target cells were added in 100 μl of IMDM+. After 6 h at 37°C, 100 μl of supernatant was removed, and the concentration of released ^{51}Cr was determined by gamma counting. The percentage of cell lysis was calculated as follows: percent specific ^{51}Cr release = [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100. Spontaneous release and total release were determined from target cells incubated with medium alone or with 1% Triton X-100, respectively.

RESULTS

The ability of vaccinia virus-RSV recombinants to induce pulmonary resistance to RSV challenge is dependent on the MHC haplotype. Previously we identified the individual RSV genes contributing to resistance to RSV challenge in BALB/c (H-2^d) mice by challenging mice previously immunized with vaccinia virus-RSV recombinants (9) at 9 and 28 days following immunization. Each protective antigen induced resistance at the early time point (day 9). In this study, we extended these observations on the ability of various vaccinia virus-RSV recombinants to induce resistance to RSV challenge in BALB recombinant mice homozygous for the H-2^d, H-2^k, or H-2^b MHC haplotype. Mice were primed i.n. and i.p. with vaccinia virus-RSV recombinants and challenged with RSV i.n. on day 9, and the level of pulmonary virus replication was assessed 4 days later (Table 1).

TABLE 1. MHC dependence of resistance induced by immunization of mice with vac-M2[a]

Mice immunized with:	Virus titer in lungs (mean log_{10} PFU/g ± SE)		
	BALB/c (H-2^d)	BALB.B (H-2^b)	BALB.K (H-2^k)
RSV	≤1.60 ± 0.00	≤1.60 ± 0.00	≤1.60 ± .00
Vac-F	≤1.60 ± 0.00	2.60 ± 0.62	≤1.60 ± 0.00
Vac-G	2.17 ± 1.01	1.85 ± 0.48	2.07 ± 0.61
Vac-M2	2.22 ± 0.80	3.90 ± 0.59	3.40 ± 0.54
Vac-N	3.04 ± 1.17	3.69 ± 0.49	3.13 ± 0.42
Vac-P	3.60 ± 0.55	4.56 ± 0.05	3.64 ± 0.49
Vac-M	3.67 ± 0.85	3.16 ± 0.62	3.98 ± 0.82
Vac-SH	4.28 ± 0.24	4.12 ± 0.53	4.32 ± 0.20
Vac-1B	4.40 ± 0.25	3.70 ± 0.58	3.53 ± 0.57
Vac-1C	4.07 ± 0.58	4.47 ± 0.25	3.28 ± 1.26
Vac-HN	3.82 ± 1.01	4.19 ± 0.49	3.90 ± 0.84

[a] Animals received vaccinia virus-RSV recombinants or RSV (10^6 PFU/ 0.05 ml) i.n. and i.p. on day 0 and were challenged i.n. with RSV (10^6 PFU/0.05 ml) on day 10. Lungs were removed for quantification of virus 4 days after challenge.

Infection with vac-F, vac-G, or RSV induced almost complete resistance to RSV challenge in mice of each of the three MHC haplotypes studied. However, the resistance induced by vac-M2 was evident only in BALB/c mice. None of the other vaccinia virus-RSV recombinants studied induced significant resistance in H-2^d, H-2^k, or H-2^b mice.

In vitro correlates of resistance to RSV challenge induced by vac-M2. Previous studies utilizing in vivo depletion of T cells with CD4- or CD8-specific monoclonal antibodies demonstrated that the protective efficacy of vac-M2 was mediated by $CD8^+$ T cells (10). In the present study, we demonstrated that the lungs of mice immunized with vac-M2 contained $CD8^+$ T cells that mediated RSV-specific cytotoxicity (Fig. 1; Table 2) in vitro. These effector cells did not lyse histoincompatible MC 57.G (H-2^b) targets infected with RSV (data not shown). The cytolytic activity of vac-M2-primed pulmonary effectors was abrogated by pretreatment with monoclonal anti-CD8 but not anti-CD4 antibodies. Thus, it was clear that the vac-M2-immunized BALB/c mice developed $CD8^+$ CTL that are known to mediate resistance to RSV (8). If pulmonary CTLs indeed are mediators of resistance to RSV infection, there should be a correlation between the presence of pulmonary CTL activity and resistance as well as between the absence of pulmonary CTL activity and susceptibility to RSV infection. This is indeed what we observed (Table 2). Separate groups of mice were immunized i.n. and i.p. with vac-M2, vac-P, vac-N, vac-1C, vac-HN (control), or RSV. Pooled effectors from lungs were obtained on day 6 and tested on BCH4 cells and uninfected fibroblasts for lysis. Pulmonary CTL activity was observed only in animals immunized with vac-M2, not vac-N or vac-P, even though the N and P proteins are known to contain CTL epitopes recognized in BALB/c mice previously infected with RSV(4, 17). Effectors from RSV-primed mice, however, showed the highest level of lysis at all effector/target (E/T) ratios tested, presumably as a result of efficient viral replication in the upper and lower respiratory tract and presentation of a wide spectrum of MHC-peptide complexes derived from various RSV proteins. We also observed that vac-M2 failed to induce pulmonary CTL activity in BALB.K mice in which resistance was not observed (data not shown). The pulmonary lymphocytes from vac-M2 primed BALB.K mice did not lyse RSV-infected L929 (H-2^k) fibroblasts, whereas those from RSV-primed mice showed 57, 47, and 20% specific lysis of these targets (data not shown). Thus,

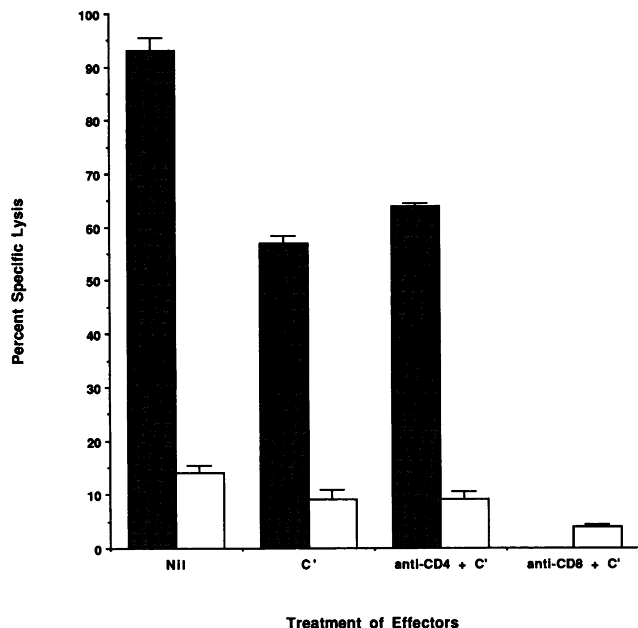

FIG. 1. RSV-specific pulmonary CTLs are $CD8^+$ T cells. The effector cell phenotype of M2-specific primary CTLs was determined on cells obtained from lung tissues on day 6. Effector cells were treated with medium (Nil) or with complement (Low Tox; Cedarlane, Ontario, Canada) alone (C'), with anti-CD4 (anti-CD4 + C'), or with anti-CD8 (anti-CD8 + C') antibodies for 60 min at 37°C. Cells were washed three times with IMDM+ before the CTL assay. E/T ratios were based on viable cell counts before the depletion procedures. Residual cytotoxic activity was assayed on BCH4 cells persistently infected with RSV (■) or uninfected BALB/c fibroblasts (□). Activation of natural killer cells, a normal feature of the early response to many viral infections (38), was not evident in the pulmonary effector cell populations obtained from vaccinia virus-primed mice on days 6, 8, 9, and 10, as tested on ^{51}Cr-labeled YAC-1 targets (data not shown).

there was a strong correlation between resistance to virus replication and the presence of pulmonary CTL activity.

Resistance to RSV challenge at various times following immunization with vac-M2 correlates with the primary CTL activity in vitro. The temporal pattern of vac-M2-induced $CD8^+$ T-cell-dependent resistance to RSV challenge was studied in order to further characterize the in vitro correlates of resistance. Previous studies demonstrated that resistance was greater on day 9 postimmunization than on day 28 (9). We expanded this information first by examining the level of resistance at earlier and later time points and second by correlating the magnitude of resistance with the level of pulmonary CTL activity. BALB/c mice were immunized with vac-M2 or vac-HN on day 0 and challenged with RSV on day 6, 10, 28, or 45, and lungs were removed 4 days later for virus quantitation. Immunization with vac-M2 induced a significant level of pulmonary resistance on day 6 (Table 3), as evidenced by a greater than 400-fold reduction in the titer of virus compared with that present in the lungs of mice immunized with the control vac-HN recombinant. Resistance of lower magnitude was evident on day 10, and there was further diminution in resistance by day 28. Resistance was virtually absent on day 45. Animals immunized with vac-HN did not exhibit restriction of RSV replication on any days tested.

TABLE 2. Correlation of the resistance to RSV challenge in mice immunized with vac-M2 with the induction of primary pulmonary CTLs

Targets	E/T ratio	Primary CTL responses (% specific lysis) of mice immunized with[a]:					
		Vac-M2	Vac-P	Vac-N	Vac-1C	Vac-HN	RSV
BCH4 cells[b]	60	37	8	1	0	0	65
	20	20	2	0	0	0	55
	6	8	0	0	0	0	49
Uninfected BALB/c fibroblasts	60	4	1	4	0	0	2
	20	4	1	1	0	0	0
	6	0	0	0	0	0	0

[a] BALB/c mice received vaccinia virus-RSV recombinants or RSV (10^6 PFU/0.05 ml) i.n. and i.p. Primary CTL responses were determined by using pooled lung effectors on day 6.
[b] BCH4-BALB/c fibroblast line persistently infected with RSV.

To examine the primary in vivo CTL responses, pooled pulmonary effector cells were obtained from BALB/c mice infected with vac-M2 (or vac-HN) 6, 8, 9, and 10 days previously. The effectors from 6-day-primed mice caused maximal lysis of BCH4 cells but not uninfected BALB/c fibroblasts at each of the E/T ratios tested (Table 3). There was a significant decline in the CTL activity of the effectors on days 8 and 9, and by day 10 there was a precipitous fall in response. Primary CTL activity was not detected on day 28 or 45.

Cytolytic ability of memory T cells generated in vitro was also examined by using splenocytes obtained from mice immunized with vac-M2 28 or 45 days previously. Although splenocytes from vac-M2-immune mice could be restimulated to produce RSV-specific CTL on day 28, the magnitude of this response was approximately half of the peak primary responses observed on day 6. By day 45 there was a sharp decline in memory CTL activity. Effectors from vac-HN-primed mice did not lyse BCH4 cells or uninfected BALB/c fibroblasts in either primary or secondary cultures. These results indicate that in the case of the M2 protein of RSV, a decrease in primary CTL activity was associated with a decrease in the level of resistance to RSV challenge and that primary, but not memory, CTL activity more closely correlates with reduction of RSV titer in lung tissue.

Evaluation of the ability of vac-M2-primed mice to clear the infection with RSV upon challenge. Results from the experiment described above indicated that low levels of memory CTL activity observed on day 45 had no detectable effect on pulmonary resistance to RSV challenge. In these experiments, the level of RSV replication was assessed on day 4, when peak virus titers are recorded in lungs of BALB/c mice (1). To examine the possibility that the residual memory CTL activity present on day 45 could facilitate early clearance of virus from lung tissues, the pattern of RSV replication in mice that had been immunized with vac-M2 45 days previously was studied. Mice were challenged with RSV as described above, and lungs were removed for quantitation of virus daily from days 2 to 10 postchallenge. Vac-M2-primed mice did not show accelerated clearance of RSV (Fig. 2). These results indicate that the residual memory CTL activity observed in the previous experiment is insufficient to accelerate clearance of RSV from vac-M2-immunized mice.

DISCUSSION

In this study, we demonstrated that immunization of *H-2^d*, *H-2^k*, or *H-2^b* mice with vaccinia virus recombinants bearing F or G surface glycoproteins induced a high level of resistance to RSV in mice of each haplotype. This finding is

TABLE 3. Correlation of the resistance to RSV challenge induced by i.n. immunization with vac-M2 with the CTL activity[a]

Days postimmunization	CTL activity	E/T ratio	CTL activity (% specific lysis)[b]				Virus titer in lungs[c] (mean $\log_{10}$ PFU/g ± SE)		Log_{10}-fold reduction[d]
			Vac-M2 primed		Vac-HN primed				
			BCH4 cells	BALB/c fibroblasts	BCH4 cells	BALB/c fibroblasts	Vac-M2 primed	Vac-HN primed	
6	Primary	60	92	15	8	5	1.7 ± 0.27	4.0 ± 0.51	2.3
		20	64	12	6	5			
		6	42	9	4	6			
8	Primary	60	37	4	7	3	ND	ND	
		20	17	3	7	1			
		6	16	5	4	0			
9	Primary	60	25	5	6	5	ND	ND	
		20	18	1	4	1			
		6	11	0	4	0			
10	Primary	60	14	0	12	0	2.6 ± 0.90	4.2 ± 0.32	1.6
		20	13	3	6	2			
		6	9	3	7	3			
28	Secondary[e]	30	45	7	7	5	3.7 ± 0.50	4.5 ± 0.40	0.8
		10	24	6	5	5			
		3	14	4	3	2			
45	Secondary	30	17	1	3	4	3.7 ± 0.60	3.7 ± 0.61	0.0
		10	9	1	4	3			
		3	5	1	1	0			

[a] Animals received vac-M2 virus (10^6 PFU) i.n. and i.p. on day 0.

[b] Primary CTL responses were determined by using pooled lung effector cells on day 6, 8, 9, and 10. Percent specific cytotoxicities of these effectors for YAC-1 natural killer cell targets were 10, 8, and 5 at the indicated E/T ratios in vac-M2-primed mice, whereas for Vac-HN-primed mice, the level of natural killer cell-mediated lysis was 11 and 6% at similar E/T ratios.

[c] Mice were challenged with RSV at 10^6 PFU/0.05 ml on days 6, 10, 28, and 45, and lungs were removed 4 days later for virus titration. ND, not determined.

[d] Reduction in replication was calculated by subtracting the mean $\log_{10}$ titer of vac-M2-infected animals from the corresponding titer of controls infected with vac-HN.

[e] Memory CTL activity was assayed on days 28 and 45 since no primary CTL activity was detectable at this time. Splenocytes from the immunized mice were restimulated in vitro by using RSV/A2 (0.5 PFU per cell) for 5 days and assayed against BCH4 cells or BALB/c fibroblasts.

consistent with previous observations that immunization with the vast majority of vaccinia virus recombinants expressing virus glycoprotein antigens readily protects animals from viral challenge (6). In contrast, the vac-M2 recombinant induced resistance only in mice of the $H\text{-}2^d$ haplotype. A surprising observation of the present study was that none of the other RSV genes studied, including the P, N, M, 1C, 1B, and SH genes, induced significant resistance in $H\text{-}2^d$, $H\text{-}2^k$, or $H\text{-}2^b$ mice. Thus, six RSV proteins in the context of seven MHC class I genes (i.e., K, D, and L genes of the $H\text{-}2^d$ haplotype and K and D genes of $H\text{-}2^k$ and $H\text{-}2^b$ haplotypes) as well as the M2 protein in $H\text{-}2^b$ and $H\text{-}2^k$ haplotypes failed to induce resistance in mice. This low frequency of responses to RSV proteins is surprising since vaccinia virus recombinants expressing lymphocytic choriomeningitis virus (LCMV) glycoprotein G or nucleoprotein (NP) induced CTL-mediated resistance in mice from at least 50% of the haplotypes tested (18). It is important to emphasize that although RSV N and P are recognized by splenic memory CTLs obtained from $H\text{-}2^d$ mice previously infected with RSV (4, 17), significant resistance to infection was not observed in the present study in BALB/c ($H\text{-}2^d$) mice immunized with a vaccinia virus-RSV N or P recombinant.

Hany et al. (18) observed several patterns of CTL responses in mice immunized with vaccinia virus-LCMV recombinants. First, immunization with vac-LCMV G or vac-LCMV N failed to induce resistance to LCMV replication in B10.BR ($H\text{-}2^k$) mice, and this failure correlated with a lack of CTL response. The majority of the vaccinia virus-RSV recombinants tested in the present study appeared to share this pattern. In the second pattern of response, a low level of CTL activity observed in $H\text{-}2^d$ mice immunized with vac-LCMV G was associated with partial resistance to virus replication (18). The duration of resistance induced in these low-responder mice was short-lived and waned rapidly by day 20 (18). This short-lived response to immunization with vac-LCMV G is similar to the response of the BALB/c mice to RSV recombinant vac-M2 seen in the present experiments. Finally, strong CTL responses were observed in $H\text{-}2^b$ mice immunized with vac-LCMV N that were long-lived (>3 to 4 weeks) and associated with restriction of virus replication and disease. This type of response was not seen in the present study. However, this pattern of response is not unique to LCMV because a vaccinia virus recombinant expressing the pp89 protein of murine cytomegalovirus also induced a long-lasting (4 months) resistance mediated by $CD8^+$ T cells in BALB/c mice (11, 20). The immunologic mechanisms underlying the differing patterns of low and high CTL responses remain uncharacterized, but there are several possibilities that could contribute to variable CTL responses, including the number of epitopes on a viral protein (6, 23), MHC class I peptide density on the antigen-presenting cells, and extent of $CD4^+$ T cell help (15).

Another explanation for the paucity of protective RSV CTL antigens identified with use of vaccinia virus-RSV recombinant virus infection of mice is that a single infection with a vaccinia virus-RSV recombinant might not be sufficient to induce a protective $CD8^+$ CTL-mediated response in the case of most viral antigens. Infection of mice or birds with a vaccinia virus-influenza virus NP recombinant was poorly protective against virus challenge (14, 37).The development of appreciable resistance required a second administration of the vaccinia virus-NP recombinant followed by challenge within a week of the booster immunization. De-

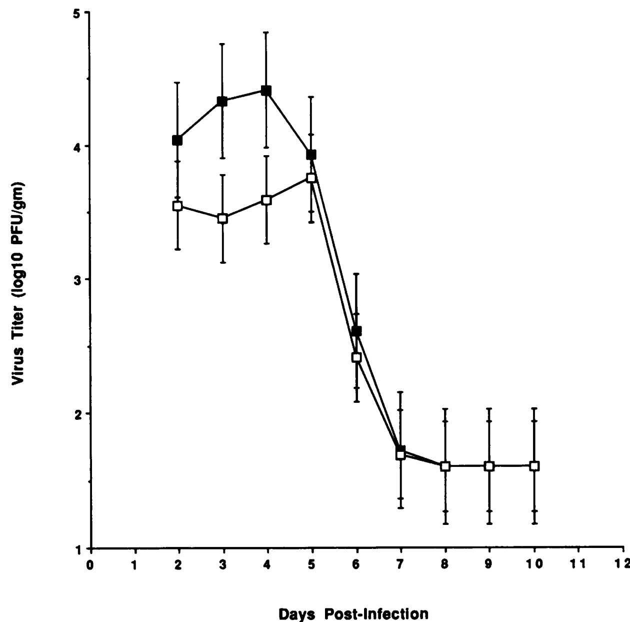

FIG. 2. Time course of RSV infection in immunized and control mice on day 45 after immunization. Animals received 10^6 PFU of vac-M2 (■) or vac-HN (□) i.n. and i.p. on day 0. Mice were challenged i.n. with 10^6 PFU of RSV per 0.05 ml on day 45, and lungs were removed for quantitation of virus daily from days 2 to 10 postchallenge.

spite this, only a moderate level of resistance (3, 37) was seen. Resistance following a booster dose of vaccinia virus-NP correlated with the presence of primary or direct CTL present at the time of virus challenge (2). It is possible that a greater number of protective RSV antigens could have been identified by using strains of mice with haplotypes other than *H-2^d^*, *H-2^k^*, or *H-2^b^* or with a two-dose schedule of immunization.

This study demonstrates that the $CD8^+$ CTL activity measured in vitro correlates with resistance observed in vivo. Three lines of evidence support this interpretation. First, the restriction of RSV replication seen following virus challenge in vac-M2-immunized mice correlated with primary pulmonary CTL activity but not with memory splenic CTL activity. Second, susceptibility to RSV challenge correlated with the absence of $CD8^+$ CTL activity in both $H\text{-}2^d$ and $H\text{-}2^k$ mice. Third, the peak of $CD8^+$ CTL activity correlated with the peak of resistance to RSV challenge. The primary and memory $CD8^+$ CTL response waned with time, as did resistance. An accelerated clearance of virus was not observed in mice challenged with RSV 45 days after immunization with vac-M2, indicating that if memory $CD8^+$ cells were present, they could not be activated sufficiently rapidly from their resting state to alter the course of RSV replication in mice. The short-lived nature of $CD8^+$ CTL-mediated resistance induced by certain viral CTL epitopes and the inability of mice with demonstrable primary CTL responses to accelerate clearance of pulmonary virus upon subsequent challenge is not a widely appreciated aspect of $CD8^+$ T-cell-mediated immunity, but it has been observed in other systems (14, 18). This weak and short-lived immunity induced by vaccinia virus-RSV M2 and vaccinia virus-influenza virus NP recombinants contrasts sharply with a strong CTL response induced by immunization with vaccinia virus recombinants bearing antigens of endogenous murine pathogens, namely, LCMV and murine cytomegalovirus, as mentioned above (11, 19).

For viral respiratory tract infections in humans, epidemiological observations indicate that the resistance mediated by cross-reacting CTLs is weak (26). The clearest example of this was seen in 1957, when the influenza A/H2N2 pandemic virus appeared in humans and caused widespread severe infections in all age groups (28). The extent of influenza illness was widespread despite the fact that the vast majority of these individuals had been infected previously more than once with H1N1 influenza A viruses bearing cross-reactive $CD8^+$ CTL determinants shared by H1N1 and H2N2 viruses (5). The H2N2 virus inherited five of the six RNA segments that coded for proteins other than the hemagglutinin or neuraminidase glycoprotein of its H1N1 virus predecessor, including the segment encoding the NP that is known to contain cross-reactive NP-specific CTL epitopes (36). Two observations from the present study facilitate an understanding to some extent of why the cross-reactive resistance in humans was so weak. First, CTL responses to certain viral antigens such as the RSV M2 protein, the LCMV G glycoprotein (18), and the influenza virus NP are of a transient nature. The level of resistance to challenge with respiratory viruses appears to correlate with primary, but not memory, CTL activity, and primary CTL activity is known to be of a transient nature (1, 2, 37, 39). Thus, the level of primary CTL activity induced by remote H1N1 virus infection was low at the time of H2N2 virus infections and therefore contributed little to resistance. Second, immunized animals with strong primary CTL responses did not manifest accelerated clearance of challenge virus administered only 45 days after immunization. Experimental observations in humans (25) and mice (14) indicate that $CD8^+$ CTLs generated from memory cell populations do not affect the peak titer of virus achieved but can have a modest effect on the clearance of virus. Since disease in humans caused by respiratory viruses correlates with the peak titer of virus present in the respiratory secretions (28), it is not surprising that memory CTLs had little impact on disease incidence. Thus, for respiratory viruses that replicate rapidly in vivo, $CD8^+$ CTLs generated from memory populations might not be able to significantly alter the pattern of viral replication. The delay in the activation of $CD8^+$ CTLs from memory populations is a function of the need for virus-infected cells to serve as antigen-presenting cells. By the time a sufficient number of $CD8^+$ T cells are generated, a large number of epithelial cells of the respiratory tract are infected, resulting in symptomatic disease. For viruses that exhibit this pattern of infection, inclusion of CTL epitopes in a vaccine probably will add little to the resistance to reinfection mediated by vaccine-induced serum and mucosal antibodies.

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