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Virus-Specific Memory and Effector T Lymphocytes Exhibit Different Cytokine Responses to Antigens during Experimental Murine Respiratory Syncytial Virus Infection

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Mice sensitized to the G (attachment) or F (fusion) glycoproteins of respiratory syncytial virus (RSV) expressed different patterns of cytokine production and lung pathology when challenged by intranasal infection with RSV. Five days after challenge, mice sensitized to G glycoprotein produced high levels of interleukin-4 (IL-4) and IL-5 in the lungs and spleens and developed extensive pulmonary eosinophilia, while mice sensitized to F glycoprotein produced IL-2 and developed a mononuclear cell infiltration. Memory lymphocytes isolated 2 weeks after intranasal challenge of mice primed to the G or F glycoprotein secreted only IL-2 and gamma interferon (IFN-γ) when stimulated with RSV. IL-4 and IL-5 production characteristic of Th2-type effectors in the lung was observed only after multiple rounds of in vitro stimulation of RSV G-specific memory T lymphocytes with antigen. Also IFN-γ production appeared to play only a minor role in the expression of pulmonary pathology characteristic of Th1 or Th2 T-lymphocyte responses, because mice genetically deficient in IFN-γ production by gene disruption displayed the same pattern of pulmonary inflammation to RSV infection after priming to RSV F or G as conventional mice. These results suggest that effector T lymphocytes exhibit a different pattern of cytokine production than memory T-lymphocyte precursors precommitted to a Th1 or Th2 pattern of differentiation. Furthermore, these observations raise the possibility that the cytokine response of human memory T lymphocytes after a single exposure to antigen in vitro may not accurately reflect the cytokine response of differentiated effector T lymphocytes at the site of infection in vivo.

T cells play a central role in the orchestration of immune responses against pathogens (17, 22, 26). Among $CD4^+$ T cells, two functional phenotypes have been defined based on their profile of secreted cytokines: Th1 T cells which secrete interleukin-2 (IL-2) and gamma interferon (IFN-γ) and Th2 T cells which secrete IL-4 and IL-5 (29, 30, 35). Results from several studies have linked certain antigenic stimuli to the preferential induction of Th1 or Th2 responses (16, 27). Of particular note are recent studies demonstrating that immunization of mice with either the respiratory syncytial virus (RSV) fusion (F) glycoprotein or the attachment (G) glycoprotein elicits $CD4^+$ T-cell responses of the Th1 or Th2 subtype, respectively, with subsequent RSV challenge (2).

RSV is the most common cause of viral lower respiratory tract infection in infants and children (23). In a murine model of RSV infection, $CD4^+$ T cells and certain T-cell cytokines, particularly IL-4 and IL-10, have been shown to participate in the generation of lung pathology when mice previously sensitized to inactivated virus are challenged with live virus (13, 21, 38). While memory Th2 T cells can be inferred to play a role in pulmonary injury after RSV infection in a vaccinated population, the temporal sequence and the dynamics of the differentiation of memory T cell precursors to activated effector cells has not been examined.

In the studies described below, we examine the T-cell responses to RSV in mice previously primed to either of the two major glycoproteins of RSV. Specifically, we analyzed the cytokines produced by RSV G or F immune effector and memory $CD4^+$ T cells during the acute and convalescent phases of infection and the concomitant pattern of lung pathology induced by RSV infection in G- or F-immune mice. Finally, we examine the contribution of one cytokine, namely IFN-γ, in dictating the differentiation of $CD4^+$ T cells and the patterns of lung injury resulting from RSV infection in IFN-γ-deficient mice. The implications of these findings for vaccination and human T-cell responsiveness are discussed.

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MATERIALS AND METHODS

Mice. Female BALB/c (*H-2*^{d}) mice age 8 to 12 weeks old were purchased from Taconic Farms, Inc., Germantown, N.Y. Mice with disrupted IFN-γ genes were bred from stock initially provided by T. Stewart, Genetech Corporation. The generation and characterization of these mice have been described previously (15). These mice were backcrossed into the BALB/C background and were homozygous for the *H-2*^{d} haplotype at the major histocompatibility complex locus. These mice were maintained in a pathogen-free condition.

Virus and infection of mice. Recombinant vaccinia virus expressing the fusion (VV-F) and attachment (VV-G) glycoproteins of RSV were obtained from J. L. Beeler (FDA, NIH). The generation and characterization of these virus have been previously described (5). Recombinant vaccinia virus expressing only the β-galactosidase (PSC11) insertion vector was used as a control. RSV (A2 strain) was a generous gift from P. L. Collins (NIAID, NIH). RSV was grown on HEp-2 cells and plaque purified under agarose as previously described (12). The virus stock was grown in HEp-2 cells and titered for infectivity. Mice were infected with 3×10^6 PFU of recombinant vaccinia virus by scarification at the base of tail. In some experiments, the mice were given 10^6 PFU of RSV in a 20-μl inoculum intranasally 3 weeks after priming and sacrificed 5 or 14 days later. Cells were isolated from spleens, lungs, and bronchial lymph nodes for in vitro culture. Lung tissue was prepared for histopathologic study.

Histopathology. Lungs of mice were harvested and fixed in 10% formalin in phosphate-buffered saline. The specimens were processed, embedded, and sectioned by American Histolabs Inc. (Gaithersburg, Md.). Sections of the lungs were prepared and stained for eosinophils by the Leinert-Giemsa technique. To compare the degree of tissue eosinophilia in the sections, we enumerated cells with characteristic eosinophil staining in and around blood vessel walls. The length of the vessels was estimated using a micrometer attached to the eyepiece of a microscope. The results were expressed as the number of eosinophils present per millimeter of blood vessel.

Lymphocyte culture. Cells were isolated from lung tissue by collagenase digestion as previously described (32). Briefly, lungs were minced in Iscove's medium (Gibco, Gaithersburg, Md.) containing 10% fetal calf serum and incubated with collagenase D (Boehringer Mannheim, Indianapolis, Ind.) at a final concentration of 0.7 mg/ml. The digestion was done at 37°C for 90 min. The digested lung tissue was tapped through a wire screen. Particulate matter was removed by a quick centrifugation at 150 × *g*. The cell suspension were layered over Ficoll-Hypaque density gradient (Lymphocyte-M; Cedarlane, Ontario, Canada) and centrifuged at 400 × *g* for 15 min. The mononuclear cells at the interface were isolated and used in in vitro culture. Single-cell suspensions isolated from spleens, lungs, or bronchial lymph node were cultured at the indicated cell numbers with irradiated naive spleen cells infected with RSV at a multiplicity of infection of 0.1. The ratio of responders to stimulators was 5:1.

Detection of cytokines in culture supernatants. Supernatants from in vitro cultures of cells isolated from spleens, lungs, or bronchial lymph nodes were collected at 48 h after stimulation and kept at −20°C until analyzed. The concentrations of IL-2, IL-4, IL-5, and IFN-γ in these supernatants were measured using commercial enzyme-linked immunosorbent assay (ELISA) reagents under conditions recommended by the manufacturer (Pharmingen, San Diego, Calif.).

Flow cytometry and fluorescence-activated cell sorting. For three-color analysis, cell pellets were incubated with fluorescein isothiocyanate-conjugated rat anti-mouse CD8, phycoerythrin-conjugated rat antimouse CD4, and tricolor-conjugated hamster anti-mouse αβ T-cell receptor or the similarly conjugated isotype-matched antibodies (Caltag Laboratories, South San Francisco, Calif.). Cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.). In some experiments, the stained cells were not fixed but resuspended at 1 million cells per ml in sterile Iscove's medium containing 10% fetal calf serum prior to isolating $CD4^+$ cells by fluorescence-activated cell sorting (FACstar flow cytometer; Becton Dickinson).

RESULTS

Pulmonary histopathology. Mice immunized with a control recombinant virus containing the PSC11 vector and challenged intranasally with live RSV showed only a mild mononuclear cell infiltration in the lung sections (Fig. 1A). This is consistent with previous observations on the histopathology of primary RSV infection in the lungs (20). In contrast, the lung sections from mice immunized with a recombinant vaccinia virus expressing either the RSV F glycoprotein (Fig. 1B) or the RSV G glycoprotein (Fig. 1C) exhibited significant inflammation (37, 40). The inflammatory cells in the lungs of VV-F-primed mice consisted primarily of mononuclear cells and some polymorphonuclear cells which had the staining characteristics of neutrophils (Fig. 1B). There was also a dense infiltration of mononuclear cell in the lung parenchyma and in the peribronchiolar and perivascular regions of VV-G-primed mice (Fig. 1C). However, unlike sections from VV-F-primed mice lung sections from VV-G-primed mice also showed extensive infiltration of eosinophils in the peribronchiolar and perivascular regions and in the lung parenchyma. Eosinophils were readily demonstrable at vascular endothelial surfaces, in vessel walls, and immediately outside the walls of vessels, indicating an active recruitment of eosinophils into the lungs of VV-G-primed mice after intranasal RSV infection (Fig. 1D). Occasional clusters of eosinophils were also found in the lungs of RSV-infected VV-F-primed animals. As shown in Fig. 2, there was significantly more perivascular eosinophil accumulation in the lungs of VV-G-primed animals than in the VV-F-primed animals after intranasal challenge with RSV.

Cytokine secretion by immune cells. In an earlier study by Alwan et al. (2) long-term T-cell lines derived from the spleens of mice immunized with VV-G secreted primarily IL-4 and IL-5 in vitro, while splenocyte lines from VV-F-primed mice produced IL-2 with little or no IL-4 or IL-5 production. In order to study the development of these functionally distinct $CD4^+$ T cells in vivo, we examined the patterns of cytokines produced by cells isolated from lungs and spleens of mice primed with either VV-F or VV-G and challenged with live RSV. Lung and spleen mononuclear cells were isolated either during an acute infection (day 5) or at day 14 when virus had been cleared. Cells from individual mice were stimulated in vitro with RSV-infected splenocyte stimulators, and the cultures from individual animals were analyzed for the production of various cytokines.

As Table 1 shows, at 5 days post-RSV infection, mononuclear cells isolated from the spleen and lungs of each RSV G-primed mouse secreted significant levels of IL-5. Lower but still significant levels of IL-5 were also detected in splenocyte cultures from RSV F-immune mice at day 5 postinfection. IL-2 production was detectable in cultures from both G- and F-primed mice but was three- to fivefold higher in mononuclear cells from F-primed mice. There was some variability of the levels of IL-4 and IL-5 production by lung mononuclear cells which correlated with the degree of pulmonary eosinophilia in the lungs of individual animals within each group. The variability of IL-5 production by lung mononuclear cells from some G-primed mice in the face of high IL-5 production in the spleens of these animals may reflect individual differences among animals in the rate of the migration of effector T lymphocytes from the spleen to the lungs at day 5 of infection. Significantly, IFN-γ was produced at high levels by cells isolated from both groups of animals.

In contrast to the findings at day 5, mononuclear cells harvested from the spleens of RSV G- and RSV F-primed mice at day 14 after intranasal RSV challenge secreted primarily IL-2 and IFN-γ after in vitro stimulation. Low and variable amounts of IL-5 and no IL-4 were produced by these immune lymphocyte populations (Table 1). The low levels of IL-4- and IL-5-producing cells in the spleens of these mice during the recovery phase of RSV infection were not readily attributed to the migration of cytokine-producing T cells to the regional lymph nodes draining the lungs, because bronchial lymph node cells from both RSV G- and F-primed mice also secreted predominantly IL-2 and IFN-γ in vitro when harvested at day 14 after intranasal RSV infection (Table 1).

Maturation of $CD4^+$ T-cell subsets in vitro. The similarity in cytokine profile between the cells harvested from RSV G-primed animals on day 5 postinfection and T-cell lines derived from spleen cells of RSV G-primed mice after multiple in vitro stimulation as reported by Alwan et al. (2) as well as the low level of IL-4 and IL-5 production by RSV G-specific T cells harvested during the convalescent phase of infection suggested the possibility that IL-4 and IL-5 production was a property of activated RSV G-specific immune effector $CD4^+$ T lymphocytes. Furthermore, the results suggested that the generation of RSV G-specific $CD4^+$ T cells with a Th2-like effector phenotype in vitro required multiple exposures to antigen. To test this possibility, splenocytes were harvested directly from RSV G-immune animals which did not receive intranasal challenge with RSV and stimulated in vitro with RSV. After 10 days in culture, $CD4^+$ T lymphocytes were positively selected by cell sorting and restimulated three more times with RSV at 10-day intervals. Supernatants were harvested 48 h after each in vitro stimulation of RSV G-primed T cells with virus.

As Fig. 3 shows, significant cytokine production was detectable in cultures of RSV G-immune T cells only after the second round of in vitro stimulation. However, these T cells produced only IL-2 and IFN-γ after this second exposure to RSV. This cytokine profile was similar to that of splenocytes taken from RSV G-primed mice at day 14 after intranasal infection. IL-2 was produced at high levels in these cultures only after the second in vitro stimulation. This effect was transient, and the levels of IL-2 in the supernatants decreased with subsequent in vitro restimulation. Detectable IL-4 production and significant levels of IL-5 were only observed after the third and fourth round of in vitro stimulation (Fig. 3).

In contrast to the results of Alwan et al. (2, 3) where RSV F

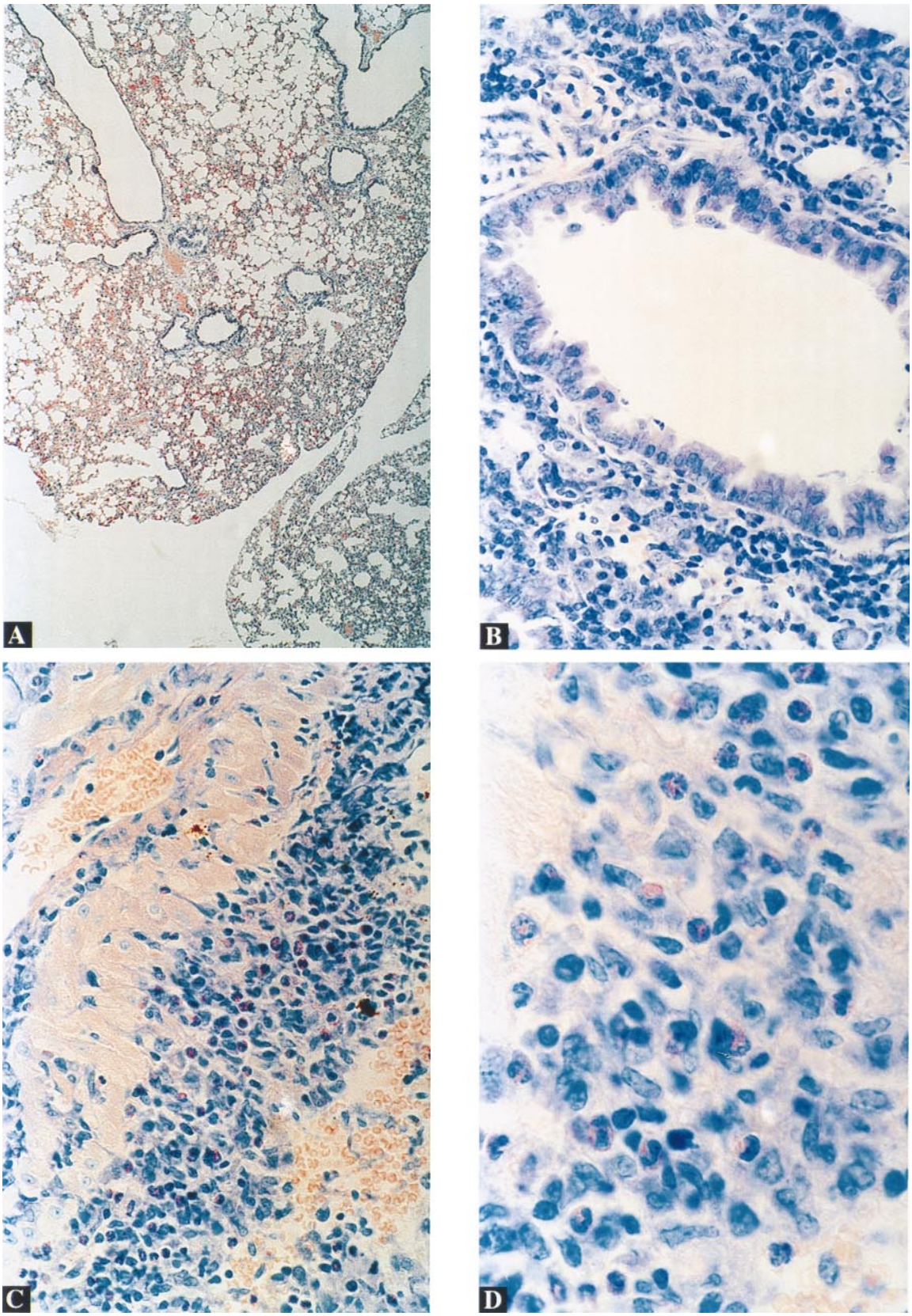

FIG. 1. Lung histopathology of mice sensitized to RSV glycoproteins. Mice were primed with control vaccinia virus (A) or recombinant virus expressing RSV F (B) or G (C and D) glycoprotein and challenged with live RSV intranasally 3 weeks after priming. Mice were sacrificed 5 days after being challenged, and lung sections were stained for eosinophils with Leinert-Giemsa's stain. Magnification: ×4 (A), ×200 (B and C), ×400 (D).

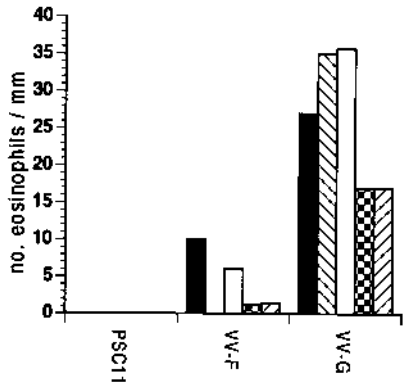

FIG. 2. Perivascular eosinophils in the lungs of mice sensitized to RSV F or RSV G glycoproteins. Mice were primed with the indicated recombinant vaccinia virus 3 weeks prior to intranasal challenge with RSV. Mice were sacrificed 5 days later, and lung sections were stained for eosinophils. Eosinophils around the blood vessels were counted, and the length of the vessels was measured by micrometer. Each column represents cell counts from individual animals. In the case of mice primed with the control vaccinia virus, no eosinophils were enumerated in the five recipients.

glycoprotein elicited a Th1 response and RSV G elicited a Th2 response, our results with short-term bulk cultures of mice sensitized to RSV F or RSV G glycoprotein and challenged with live RSV showed a more complex pattern of cytokine secretion with considerable overlap between these two groups of animals. To clarify this issue, we generated $CD4^+$ T-cell lines from spleens of mice primed with RSV F or RSV G. Table 2 shows the cytokine profiles of splenocyte cell lines derived from two RSV F- and two RSV G-immune mice after the fourth in vitro stimulation with RSV. In agreement with the results of Alwan et al., F-specific T-cell lines produced higher levels of IL-2 and lower levels of IL-4 and IL-5 than did G-specific T-cell lines. However, T-cell lines derived from either F- or G-primed animals secreted high levels of IFN-γ in response to stimulation with RSV-infected spleen cells. This is consistent with the results from the short-term bulk cultures and suggests that a strong Th1-like IFN-γ response is demonstrable in both G- and F-primed animals.

IFN-γ and pulmonary eosinophil infiltration. An unexpected result in our analysis was the finding that high levels of IFN-γ were produced by lung and spleen cells from RSV G-primed mice in the face of high levels of the Th2 cytokines IL-4 and IL-5. Several studies have previously demonstrated the simultaneous production of IFN-γ and IL-4 during experimental viral pneumonia (6, 28). However, in these studies the animals were either naive or previously primed with intact virus. Under these conditions, lung pathology indicative of a preferential Th2-like response would not be expected during viral infection. Although IFN-γ has been implicated as an important negative regulator of Th2 differentiation (8, 34), the findings reported here raise the possibility that IFN-γ is not an important factor in the regulation of IL-5 production and pulmonary eosinophil infiltration during experimental RSV infection.

To further explore this issue, we examined the histologic changes in the lungs of RSV-infected mice rendered IFN-γ deficient by targeted gene disruption (15). IFN-γ-deficient mice were primed with VV-G or VV-F and subsequently challenged intranasally with infectious RSV. If IFN-γ produced in VV-F-primed mice after RSV infection played a major role in inhibiting Th2 cytokine production and pulmonary eosinophilia then IFN-γ-deficient mice previously primed with RSV F would, like RSV G-immune animals, demonstrate enhanced pulmonary eosinophil accumulation in response to intranasal RSV challenge. This was not the case.

Figure 4 shows representative histologic sections from the lungs of mice primed with either VV-G, VV-F, or a control vaccinia virus and challenged intranasally with infectious RSV 3 weeks later. Lungs were removed and examined histologically 5 days postinfection. Overall, the histologic changes in the lungs of the IFN-γ-deficient mice after RSV infection were similar to findings in conventional mice with an intact IFN-γ response. Lungs of IFN-γ-deficient mice primed with the control vaccinia virus showed a mild peribronchial and perivascular infiltrate of mononuclear cells after RSV challenge (Fig. 4A). Sections of lungs from RSV F-primed IFN-γ-deficient mice showed, like infected lungs from RSV F-primed conventional mice, a dense peribronchiolar and perivascular infiltration of mononuclear inflammatory cells in response to RSV challenge, with few eosinophils evident in the sections (Fig. 4B). Thus, prior exposure to RSV-F in IFN-γ-deficient mice did not enhance the accumulation of eosinophils in the lungs of these animals in response to RSV infection. In contrast, IFN-γ-deficient mice primed with RSV G did show a marked infil-

TABLE 1. Cytokine production by immune cells during acute RSV infection or after RSV infection[a]

Mononuclear cell source	Mice primed with:	Cytokine production (pg/ml)			
		IL-2	IL-4	IL-5	IFN-γ
Day 5					
Spleen	VV-F	565 (63)	45 (10)	295 (127)	6,922 (493)
	VV-G	110 (10)	19 (7)	881 (141)	12,510 (1,103)
Lung	VV-F	287 (147)	55 (15)	741 (186)	11,878 (3,631)
	VV-G	168 (13)	201 (43)	4,540 (889)[b]	24,686 (3,618)
Day 14					
Spleen	VV-F	314 (14)	9 (9)	16 (7)	24,947 (1,445)
	VV-G	427 (79)	14 (8)	83 (17)	6,375 (318)
Lung	VV-F	ND[c]	ND	ND	ND
	VV-G	238 (20)	12 (5)	73 (24)	3,751 (591)
Bronchial lymph node	VV-F	62 (13)	24 (9)	88 (46)	21,603 (1,910)
	VV-G	59 (5)	24 (17)	232 (108)	9,086 (3,515)

[a] Groups of mice ($n = 4$) were scarified with VV-F or VV-G. Three weeks after priming they were challenged with live RSV intranasally. Mice were sacrificed 5 or 14 days later, and cells were isolated from spleens, lungs, and bronchial lymph nodes and stimulated in vitro with RSV. Values are mean (standard error of the mean) cytokine production of cultures from four individual mice per group except as noted.

[b] Mean (standard error of the mean) IL-5 values were obtained from three animals. A lung IL-5 level of 622 pg/ml from one animal was excluded from this calculation.

[c] ND, not done.

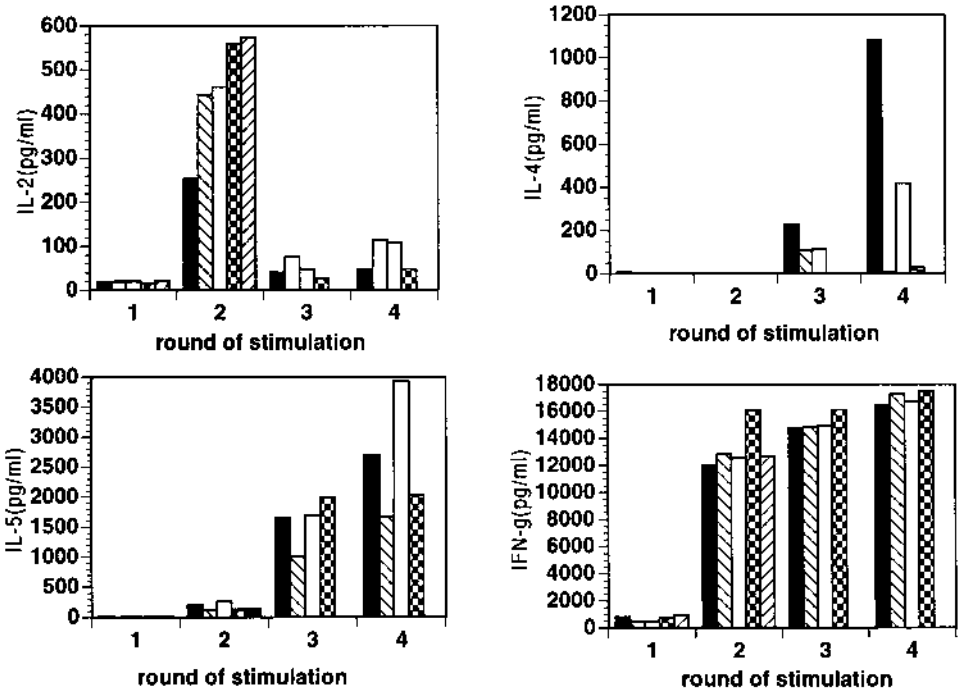

FIG. 3. Kinetics of in vitro maturation of cytokine-producing cells. Spleen cells from mice primed with VV-G were stimulated with RSV-infected naive spleen cells. After the first stimulation, $CD4^+$ cells were isolated by fluorescence-activated cell sorter and stimulated every 10 days with RSV-infected spleen cells. Supernatants were collected 48 h after the indicated passage and analyzed for cytokine production by ELISA.

tration of mononuclear cells and eosinophils in their lungs when challenged with infectious RSV (Fig. 4C). Quantitative morphometry of the perivascular eosinophil numbers in the lungs of vaccinia virus control and VV-F- and VV-G-primed IFN-γ-deficient mice confirmed these interpretations (Fig. 5).

DISCUSSION

In this report we have examined the spectrum of histopathology and the character of the cytokine response in the lungs of BALB/c mice primed to either the RSV G or F glycoprotein and subsequently challenged intranasally with infectious RSV. In keeping with the findings of an earlier report (31), we found that prior exposure to RSV G (delivered in a recombinant vaccinia virus) predisposes the mice to develop upon subsequent RSV infection a pulmonary infiltrate characterized by extensive eosinophil accumulation. After priming with RSV F, on the other hand, RSV-infected mice show predominantly a mononuclear cell infiltrate with few eosinophils in lung sections.

The difference in the character of the lung histopathology in mice immune to F or G is most easily attributable to a quantitative rather than qualitative difference in the array of cytokines produced by effector T lymphocytes generated during infection of F- or G-primed mice with RSV. Consistent with this view we found that lung and spleen mononuclear cells isolated from G-primed mice at day 5 after RSV infection produced higher levels of IL-4 and IL-5 than mononuclear cells from infected RSV F-primed mice. There was variability from animal to animal among G-primed mice in the extent of lung eosinophil accumulation, which correlated with the magnitude of IL-5 production by lung mononuclear cells from individual G-primed mice. As noted above, these differences from animal to animal in lung histopathology and lung and spleen cytokine profile among G-primed mice may reflect individual variability in the tempo of recruitment of activated effector T lymphocytes from the spleen to the site of infection in the lungs.

In a striking contrast to the cells isolated from mice during an acute phase of RSV infection, mononuclear cells isolated 14

TABLE 2. Cytokines produced by RSV G- and RSV F-specific T-cell lines[a]

T-cell line and specificity	Concentration (pg/ml)				Cell surface phenotype (%)	
	IL-2	IL-4	IL-5	IFN-γ	αβ T-cell receptor	CD4
G line 1	46	1,080	2,968	16,410	100	100
G line 2	108	836	3,934	17,260	100	100
F line 1	1,042	214	854	15,750	100	100
F line 2	906	114	1,814	15,770	100	100

[a] T-cell lines were derived from spleen cells of individual mice primed with VV-F or VV-G. Spleen cells were stimulated with irradiated RSV-infected spleen cells from unimmunized animals at a cell density of 1 million cells per ml every 10 days. Supernatants were collected 48 h after the fourth in vitro stimulation and analyzed for cytokines by ELISA. Data are representative of three independent experiments in which RSV G- and RSV F-specific lines were generated from individual VV-G- or VV-F-primed mice.

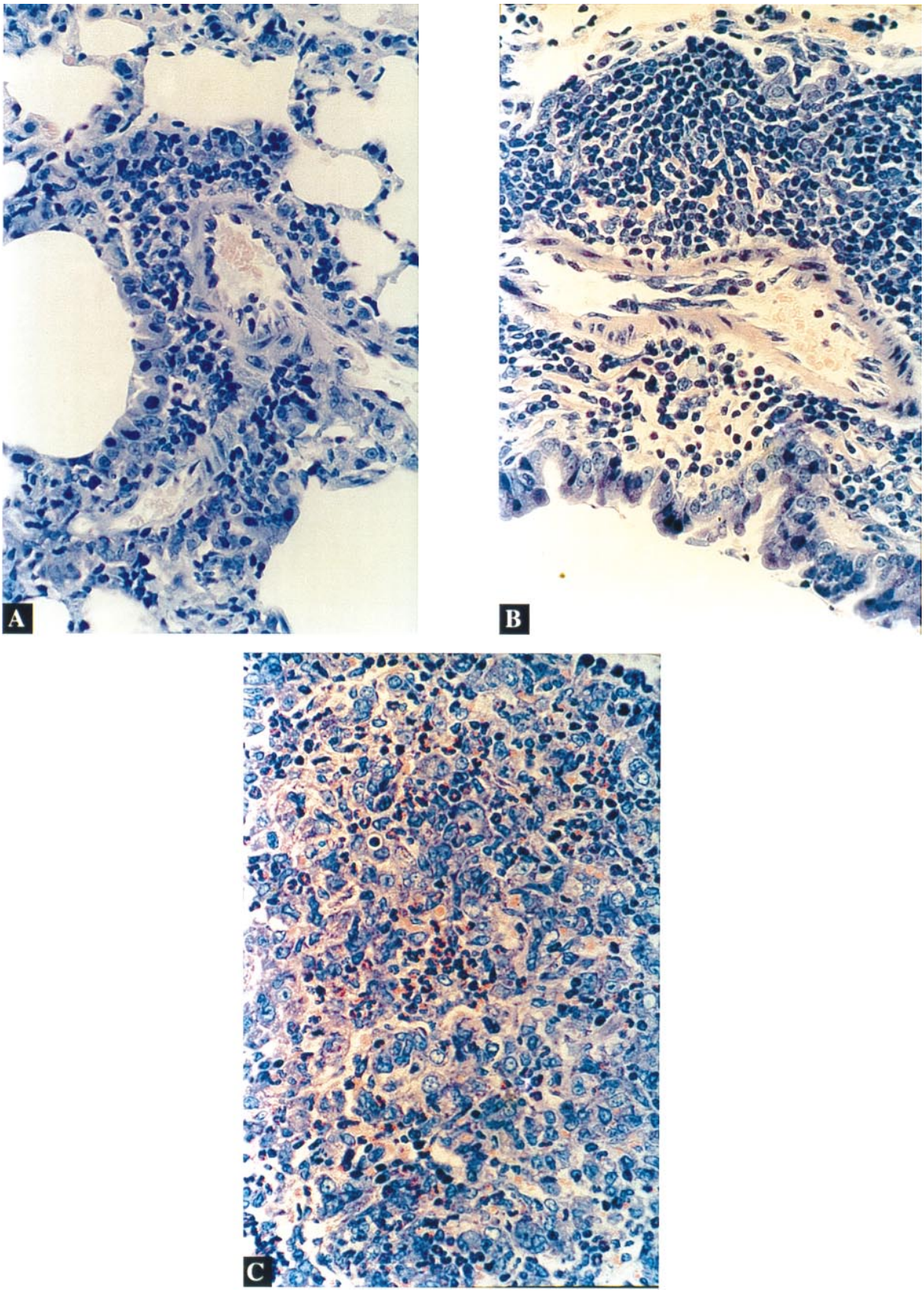

FIG. 4. Lung histopathology of IFN-γ-deficient mice sensitized to RSV glycoproteins. Mice were primed with control vaccinia virus (A) or recombinant vaccinia viruses expressing RSV F (B) or G (C) glycoprotein and challenged with live RSV intranasally 3 weeks after priming. Mice were sacrificed 5 days after being challenged, and lung sections were stained for eosinophils with Leinert-Giemsa's stain. Magnification, ×200.

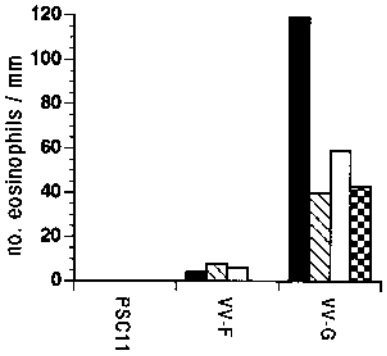

FIG. 5. Perivascular eosinophils in the lungs of IFN-γ-deficient mice sensitized to RSV F or RSV G glycoprotein. Mice were primed with the indicated recombinant vaccinia virus 3 weeks prior to intranasal challenge with RSV. Mice were sacrificed 5 days later, and lung sections were stained for eosinophils. Eosinophils around the blood vessels were counted, and the lengths of the vessels were measured by micrometer.

days after intranasal challenge of mice primed against either of the two major glycoproteins of RSV secreted predominantly IL-2. Thus, memory T lymphocytes taken during the convalescent phase of infection exhibit a different cytokine profile than effector cells taken during the acute phase of infection. This cytokine pattern is similar to that of memory T lymphocytes isolated from mice primed with VV-G without in vivo exposure to RSV. These day 14 memory T lymphocytes display a cytokine profile similar to that of active (day 5) effector cells, i.e., high IL-5 and low IL-2 production only after several rounds of in vitro restimulation of the memory T cells with RSV. The absence of IL-4 and IL-5 production during the first round of in vitro stimulation of memory T lymphocytes could be attributed to the production of IFN-γ by the stimulated memory cells. IFN-γ might then inhibit the production of IL-4 and IL-5 during in vitro stimulation. However, this seems unlikely since the effector cells isolated from animals during an acute RSV infection as well as the effector cells generated in vitro after multiple stimulation produce high levels of IL-4 and IL-5 in the presence of even higher levels of IFN-γ. Also coculture of RSV G-primed T cells with RSV in the presence of neutralizing anti-IFN-γ antibody had no effects on the timing of IL-4 and IL-5 production (unpublished observation).

Our findings are in keeping with the results of Bradley et al. (10), who reported that memory $CD4^+$ T lymphocytes primed to a soluble protein antigen produce IL-4 and IL-5 only after in vivo reexposure to the antigen. In that study with memory $CD4^+$ T cells as well as in several reports using naive or memory $CD4^+$ cells (9, 18), $CD4^+$ T-cell populations produce IL-2 and low levels of IFN-γ upon the first encounter with antigen in vitro. Production of Th2-like cytokines by $CD4^+$ T cells required multiple exposures to antigen in vitro. In this regard, it is noteworthy that the Th2-like phenotype exhibited by splenic T-lymphocyte culture from RSV G-primed mice in the study of Alwan et al. (2) was observed in long-term cell lines maintained by repeated in vitro stimulation with RSV. Their observation as well as the findings reported here raises the possibility that in contrast to active effector cells, RSV-specific memory $CD4^+$ T cells committed to a Th1 or Th2 pattern of differentiation require multiple exposures to antigen to express the cytokine profile characteristic of the Th1 or Th2 subset. Repeat exposure would normally occur in vivo during the acute phase of RSV infection when active virus replication provides a continuous supply of antigen-presenting cells expressing G or F antigenic epitopes. Whether the absence of a Th2-like cytokine response in G-primed mice during the late convalescent phase of RSV infection reflects terminal differentiation of effector cells leading to activation-induced cell death (36, 39) or reversion of these $CD4^+$ T cells to a memory phenotype awaits experiment proof.

IFN-γ has been reported to play an important regulatory role in the differentiation of $CD4^+$ T cells along the Th1 pathway (8, 19, 34). It was therefore noteworthy that mononuclear cells from G-primed mice produced high levels of IL-4 and particularly IL-5 during the acute phase of infection when IFN-γ production was also high. These findings suggested that IFN-γ production by T lymphocytes (or natural killer cells) early in response to RSV infection does not influence the subsequent differentiation of RSV-specific $CD4^+$ T cells into effector cells of the Th1 or Th2 subset. Rather it is the initial exposure of naive T cells to either RSV F or G during the primary response which appears to dictate the cytokine response of effector T cells which derives from these memory $CD4^+$ T cells after RSV challenge. In keeping with this concept, we found that the overall pattern of inflammation in the lungs of RSV G- or F-primed IFN-γ-deficient mice was comparable to that of conventional mice with an intact IFN-γ response. In particular, the finding that RSV F-primed IFN-γ-deficient mice did not show a significant increase in pulmonary eosinophil accumulation after RSV challenge argues for factors other than IFN-γ production dictating the cytokine production and inflammatory response in the lungs of RSV G- or F-primed mice. This is in agreement with previous reports in which a short-term in vivo blockade of IFN-γ did not switch the response to the Th2 phenotype (7, 33). It should be noted, however, that eosinophil accumulation was greater in the lungs of G-primed IFN-γ-deficient mice than in G-primed conventional mice. Perhaps while IFN-γ does not play a prominent role in regulating the induction and differentiation of IL-5-secreting T cells directed to RSV G, IFN-γ may modulate the extent of tissue eosinophilia by inhibiting the migration of $CD4^+$ Th2 effector T cells to the sites of antigen accumulation (24).

The presence of peripheral blood eosinophilia and eosinophils in the lungs of individuals who had been immunized with formalin-inactivated RSV and subsequently infected with the virus raises the possibility that RSV-specific $CD4^+$ Th2 T cells may in part orchestrate this distinct pattern of lung pathology (11, 25). Our results as well as the observations of other investigators (1, 13, 14, 21) support this view and indicate a direct correlation between the presence of $CD4^+$ effector T cells of Th1 or Th2 phenotype in the lungs and type of lung injury observed in mice after RSV challenge. The results reported here further suggest that multiple rounds of antigen exposure may be required to generate Th2 effector cells from antigen-specific memory precursor cells. In support of this concept, a recent analysis of cytokine gene expression in RSV-immune individuals revealed that their peripheral blood mononuclear leukocytes expressed primarily IL-2 and IFN-γ in response to one round of in vitro stimulation with RSV (4). Our data also suggest that modifying this differentiation process by exogeneously administered cytokines or neutralization antibodies specific to cytokines would modify the course of disease. In keeping with this concept, it has been shown that the depletion of IL-4 and IL-10 during RSV infection in mice primed with formalin-inactivated RSV resulted in significant reduction in peribronchiolar and perivascular inflammatory infiltrates (13). Further insight into the mechanisms by which antiviral T cells differentiate into either memory precursor cell or active effector cells will be necessary for the rational design of immunoprophylactic agents against this virus.

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