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In Vivo Blockade of Gamma Interferon Affects the Influenza Virus-Induced Humoral and the Local Cellular Immune Response in Lung Tissue

NICOLE BAUMGARTH* AND ANNE KELSO

Cooperative Research Center for Vaccine Technology and Transplantation Biology Unit, Queensland Institute of Medical Research, Brisbane, Queensland, Australia

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Influenza virus infection induces the local production of gamma interferon (IFN-γ) by T cells and non-T cells in the respiratory tract. To elucidate the possible functions of this cytokine, the humoral and local cellular immune responses to influenza virus were studied in BALB/c mice with or without in vivo neutralization of IFN-γ by using monoclonal antibodies. Neutralization of IFN-γ led to a significant reduction in virus-specific titers of immunoglobulins G2a and G3 in serum but had little effect on other isotypes. Studies on cells isolated from the lung parenchyma itself revealed that at the height of the immune response the ability of these cells to produce cytokines after antigen or T-cell receptor/CD3 stimulation was not affected. Ex vivo cytolytic activity by lung parenchyma cells, which is induced by infection with this virus in normal mice, was also found to be undisturbed by this treatment, even though anti-IFN-γ antibody activity was recovered from lung lavage samples and sera at all days studied. Surprisingly, in vivo neutralization of IFN-γ led to a significant reduction in the magnitude of the cellular infiltrate in the lung tissue which followed infection, suggesting an involvement of IFN-γ in the mechanisms that regulate increased leucocyte traffic in the inflamed lung parenchyma. This conclusion was supported by findings of differences between mock-treated and anti-IFN-γ-treated mice in the number of $CD8^+$ lung T cells expressing CD49d (α4-integrin) and CD62L at various times after influenza virus infection. This study therefore demonstrates that IFN-γ affects the local cellular response in the respiratory tract as well as the systemic humoral response to influenza virus infection.

One of the hallmarks of influenza virus infection and of viral infections in general is the induction of type I and type II interferon production by a wide range of cell types (27). Type I interferons produced by cells following virus invasion exhibit their antiviral activity through the induction of various interferon-regulated proteins which cause the inhibition of virus replication. Gamma interferon (IFN-γ), on the other hand, has been identified as an important immunoregulatory cytokine. It is produced by T cells, NK cells, and macrophages and has pleiotropic effects on various cells of the immune system (8, 37). Earlier reports suggested that this cytokine is necessary for the development of $CD8^+$ T cells into effector cytotoxic T lymphocytes (CTL) in vitro and for induction of lymphocytic choriomeningitis virus-specific CTL in vivo (20, 31, 41). Recent experiments with IFN-γ and IFN-γ receptor gene-targeted mice, however, have shown that the induction of precursor CTL in the spleens of influenza virus-infected mice (10) and the effector CTL response in the spleens of vaccinia virus-infected mice were not altered (14), whereas the CTL response against lymphocytic choriomeningitis virus infection was only slightly reduced (14). Experiments with these gene-targeted mice showed that a crucial mechanism for the antiviral activity of IFN-γ is the activation of macrophages leading to their production of nitric oxide synthase. Inhibition of ectromelia virus, vaccinia virus, and herpes simplex virus type 1 replication in mouse macrophages correlated with the cells' production of nitric oxide (17). IFN-γ is also reported to influence humoral immune responses. It was shown to enhance in vivo the induction of *Brucella abortus*-specific immunoglobulin G2a (IgG2a) (7), an Ig isotype which is superior to IgG1 in binding of complement (23), in promoting natural killer cell-mediated lysis of target cells (16), and in binding to the high-affinity Fc receptor on macrophages (38). Furthermore, IFN-γ receptor gene-targeted mice were reported to have reduced total levels of IgG2a in serum and, after immunization, reduced levels of antigen-specific IgG2a and, to a lesser extent, reduced levels of IgG3 (14).

Other effects of IFN-γ defined in vitro that could have a bearing on the regulation of the local immune response are the changes induced in endothelial cells. IFN-γ stimulates the expression of surface major histocompatibility complex class II molecules and mouse endothelial cell antigen (MECA) 325 antigen on endothelial cells (6, 26). The expression of the MECA 325 antigen is usually restricted to the specialized high endothelial venules (HEV), which can facilitate lymphocyte migration. IFN-γ has also been shown to enhance the antigen-presenting capacity of endothelial cells (9) and to increase their ability to bind T and B cells (42). In vivo IFN-γ has been reported to increase vascular permeability (21) and stimulate lymphocyte migration into the skin and T-cell recruitment into delayed-type hypersensitivity reactions (15).

Most reports on the local immune response to experimental influenza virus infection in mice have studied the bronchoalveolar lavage (BAL) fluid in which high levels of IFN-γ have been found early during the infection (13, 35). We have previously reported (2) that both T cells and non-T cells in the lung parenchyma itself increase their IFN-γ mRNA expression and their ability to secrete IFN-γ protein in a virus-dose-dependent manner following influenza virus infection; by comparison, T cells in the draining mediastinal lymph nodes (MLN) express lower levels of IFN-γ mRNA and have a lower

* Corresponding author. Mailing address: Queensland Institute of Medical Research, Post Office, Royal Brisbane Hospital, Queensland 4029, Australia. Phone: 61-7-33620379. Fax: 61-7-33620105. Electronic mail address: nicoleB@qimr.edu.au.

capacity to produce this cytokine after restimulation in vitro. Given the intensity of the IFN-γ response in the respiratory tract, it seems likely that IFN-γ would play some part in the immune response to influenza virus infection in this effector site. To identify this role, we investigated the effects of IFN-γ neutralization with monoclonal antibodies (MAb) both on the systemic humoral response and on the local cellular response in the draining lymph nodes and the lung parenchymas of normal mice after influenza virus infection. Surprisingly, the dominant function for IFN-γ revealed by this approach was in the recruitment and/or retention of leucocytes in the lung parenchyma.

MATERIALS AND METHODS

Animals and virus. Six- to eight-week-old female BALB/c mice were obtained from the Animal Resources Centre, Perth, Australia, and held in filter-top cages at the Queensland Institute of Medical Research. Mice were anesthetized with ether and infected intranasally (i.n.) with 10 hemagglutinating units (HAU) of influenza virus in a total of 50 μl of phosphate-buffered saline (PBS) (8×10^5 PFU per mouse). The virus used was a reassortant, Mem71, bearing the hemagglutinin of A/Memphis/1/71 (H3) and the neuraminidase of A/Bellamy/42 (N1) and was harvested and stored as described before (2).

Antibody treatment. A total of 3 mg of the anti-IFN-γ MAb XMG 1.2 (5) or 3 mg of rat Ig (Jackson ImmunoResearch Lab., West Grove, Pa.) was injected intravenously (i.v.) into mice at days −1, 0, and 3 in relation to infection with virus. The anti-IFN-γ MAb was purified from hybridoma supernatants containing 5% fetal calf serum (FCS) by using a protein G column (gammaBind G; AMRAD Pharmacia Biotech, North Ryde, Australia) and stored in aliquots at −20°C. After purification, the antibody preparation was shown to have retained its ability to inhibit the antiproliferative effects of recombinant IFN-γ (rIFN-γ) on the cell line WEHI-279.

Cell preparation and lung digest. Mice were killed by cervical dislocation, and the lungs were flushed in situ with 20 ml of balanced salt solution via cannulation of the heart to remove the intravascular blood pool. Minced lung tissue was incubated for 90 min at 37°C on a rocker, in Dulbecco's modified Eagle medium modified as described elsewhere (18) (10 ml per three lungs) and supplemented with 10% FCS, 216 mg of L-glutamine per liter, 5×10^{-5} M 2-mercaptoethanol and antibiotics, DNase I (50 U/ml; Boehringer, Mannheim, Germany), and collagenase I (250 U/ml; type 4197; Worthington, Freehold, N.J.). Passing the digested lung tissue through a stainless steel mesh and subsequently performing discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) centrifugation (35 to 55%) yielded purified live lung parenchymal cells. Single-cell suspensions from MLN draining the lower respiratory tract were prepared in mouse tonicity, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered balanced salt solution supplemented with 2.5% heat-inactivated FCS (Cytosystems, Castle Hill, Australia) by passing tissues through a stainless steel sieve and removing erythrocytes by NH_4Cl lysis.

BAL. BAL was performed to confirm that neutralizing anti-IFN-γ MAb were present in the lungs. Skin and muscle tissues ventral to the trachea were removed carefully so as not to disrupt any blood vessels. A small incision in the trachea immediately caudal to the larynx was made, a blunt-ended 19-gauge needle attached to a 1-ml syringe was inserted, and the lungs were lavaged four times with 1 ml of PBS. Samples from individual mice were centrifuged, and the supernatants were kept at −70°C until tested for anti-IFN-γ activity by bioassay, using the cell line WEHI-279 (see below).

Cell staining and flow cytometry. Cell staining was done for 20 min on ice, at a cell concentration of 10^6 cells per 25 μl of cold balanced salt solution with 5% FCS and antibodies. Phycoerythrin-conjugated anti-mouse CD4 MAb (GK1.5; Becton Dickinson) and fluorescein isothiocyanate-conjugated anti-mouse CD8 MAb (53-6.7, Becton Dickinson) were used to stain T-cell populations. Supernatants from hybridoma KT3 (anti-CD3), RB3-6B2 (anti-B220), M1/70.15 (MAC-1α), F4/80, N418 (dendritic cell marker), and Mel-14 (anti-CD62L) as well as purified anti-CD49d (R1-2; Pharmingen, San Diego, Calif.) were used in separately determined optimal concentrations with mouse adsorbed rabbit anti-rat Ig-fluorescein isothiocyanate (Vector, Burlingame, Calif.) as second-step reagent. Biotinylated anti-CD45RB (23G2) and anti-CD44 (IM.7) (both from Pharmingen) were used at previously determined optimal concentrations with streptavidin-fluorescein isothiocyanate (Becton Dickinson) as detection reagent. As negative controls, cells were incubated with the second-step reagent only, since in previous experiments at the determined optimal concentration of the first reagent no shift was observed when isotype-matched control antibodies were employed. Propidium iodide was added last to the suspension of stained cells at 1 μg/ml. Cell populations were analyzed by using a FACScan (Becton Dickinson) with the Lysis II software program after logical gating on lymphocyte forward and side scatter and exclusion of propidium iodide-stained cells.

Cytokine production in vitro. Single-cell suspensions obtained from the MLN and lung parenchymas of individual mice were cultured at 10^6 cells per ml of Dulbecco's modified Eagle medium for 24 h in anti-CD3-coated 24-well tissue culture plates, and supernatants were analyzed for the presence of interleukin-2 (IL-2), IL-4, IL-5, and IFN-γ as described elsewhere (2, 18). For the determination of virus-specific cytokine production, lung tissue cells were cultured for 24 h in flat-bottom 96-well plates containing 2.5×10^5 irradiated spleen cells per well which were pulsed for 90 min with 600 HAU of Mem71 or allantoic fluid from noninfected eggs. Supernatants from virus-stimulated cultures showed some growth inhibition of the cell line WEHI-279 which was not reversed with the anti-IFN-γ MAb R4-6A2. IFN-γ activity was therefore determined by subtracting the calculated levels of IFN-γ in the presence of R4-6A2 from those obtained in its absence. To determine the levels of anti-IFN-γ activity in body fluids from individual mice, sera and lavage samples were tested for their ability to inhibit the antiproliferative capacity of rIFN-γ, expressed as the reciprocal titers that led to a 50% inhibition of 3 U of rIFN-γ activity per ml, added at 3 U/ml, on the cell line WEHI-279.

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates (96-well; Dynatech Laboratories, Chantilly, Va.) were incubated for 16 h at room temperature with 1,000 HAU of influenza A virus Mem71 (kindly provided by L. Brown, University of Melbourne, and J. Bates, CSL Ltd.) per ml in coating buffer (0.2 M sodium bicarbonate buffer, pH 9.6). After being washed in PBS-Tween and PBS, plates were incubated for 4 h or overnight with mouse serum stepwise diluted in PBS containing 1% FCS, 0.1% (wt/vol) dried milk powder, and 0.05% Tween (blocking buffer). Ig binding was detected with biotinylated goat anti-mouse IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 antibodies (Southern Biotechnologies Assoc., Birmingham, Ala.) and streptavidin-horseradish peroxidase (Vector) at a previously determined optimal concentration in blocking buffer, each incubated for 2 h. Enzyme activity was detected with the substrate 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis, Mo.) and read at dual wavelengths (415 nm and 490 nm) with a microplate reader (Bio-Rad, North Ryde, Australia). A hyperimmune serum was prepared as a standard by injecting mice with 500 HAU of Mem71 (intraperitoneally) and giving them boosters with the same amount 4 weeks later. Serum samples were taken 7 days after the boosters were given and were stored in aliquots at −20°C. A dilution series of this serum at a starting concentration of 1/1,000 for the detection of IgA and 1/4,000 for the detection of all other Igs generated a standard curve. The point on the steep part of the curve that gave an optical density of 1.0 was arbitrarily defined as 100 antibody units. Antibody units of mouse sera were then calculated by comparison of their dilution series with the standard curve.

^{51}Cr release assay. The mastocytoma cell line P815 (*H-2^d*) was labelled with 100 μCi of $Na^{51}CrO_4$ (Amersham Corp., Baulkham Hills, Australia) by incubation for 90 min at 37°C in 5% CO_2 in the presence of either 180 HAU of Mem71, allantoic fluid from noninfected eggs, or medium only. Graded numbers of respiratory tract effector cells were cultured in triplicates in the presence of 10^4 labelled target cells for 6 h at 37°C in 5% CO_2. A total of 100 μl of supernatant was than taken and measured using a gammacounter (Compugamma; LKB, Thornleigh, Australia). Percent specific lysis was determined by the following formula: 100(test release − spontaneous release) ÷ (total release − spontaneous release).

Statistics. Statistical significance was determined by Student's *t* test ($P < 0.05$).

RESULTS

Anti-IFN-γ treatment of influenza virus-infected mice. The influenza A virus strain Mem71 was chosen for the following studies because this strain grows to high titers in the lungs of infected BALB/c mice (3a) before being cleared. Preliminary comparative studies revealed no difference in the induction of the cellular immune response in the lungs of BALB/c and C57/BL6 mice, and BALB/c mice were used for all studies presented here. We have previously shown that IFN-γ was strongly induced in the lung parenchyma of this mouse strain after influenza virus infection (2). To investigate its effects on the humoral response and the local cellular response within the respiratory tract, we neutralized IFN-γ activity in vivo, using anti-IFN-γ MAb. In three independent experiments, mice were treated i.v. either with anti-IFN-γ MAb or with rat Ig and were then infected with influenza virus. At days 0, 2, 4, 6, and 10 after infection, three mice from each group were killed and sera and tissues were taken for analysis. In one experiment, lavage samples from individual mice of both groups were also taken. Mice from both groups developed signs of clinical illness between days 3 and 6 and recovered from infection at a similar rate. Macroscopic investigation of the lungs after infection nevertheless revealed some differences between the two groups, in that mice pretreated with anti-IFN-γ developed patchy lung consolidations by day 4 whereas in the lungs of

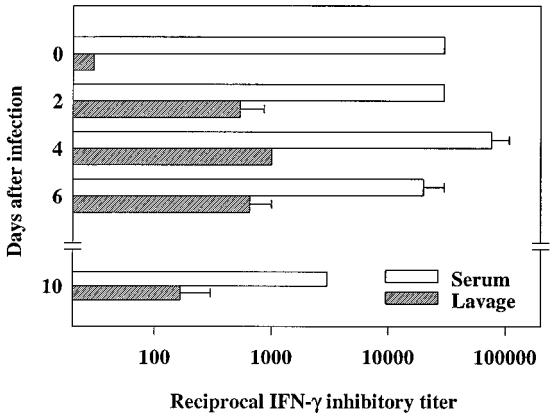

FIG. 1. Levels of neutralizing anti-IFN-γ activity in sera and BAL samples of mice treated with the anti-IFN-γ MAb XMG1.2. Sera and BAL samples from individual mice at indicated times after i.n. infection with 10 HAU of influenza virus were tested for their ability to inhibit the activity of IFN-γ on the cell line WEHI-279. Shown are the reciprocal titers of the body fluids that led to 50% inhibition. No activity was found in sera from rat Ig-treated mice (data not shown). Error bars, standard deviations.

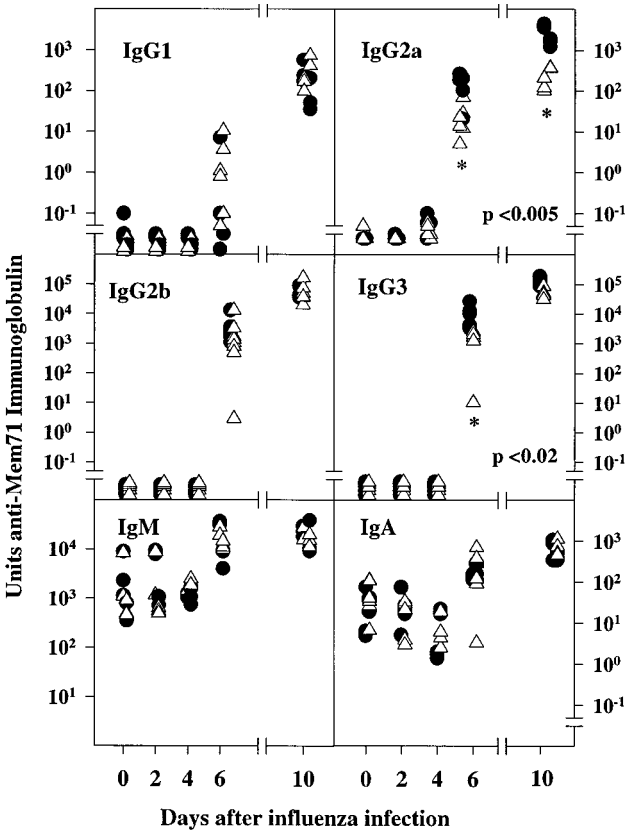

FIG. 2. Effects of anti-IFN-γ treatment on anti-influenza virus antibody levels in serum. In three independent experiments mice were i.v. treated with rat anti-IFN-γ MAb (triangles) or with rat Ig (circles). Serum samples from individual mice were analyzed by ELISA for influenza virus-specific Ig. Breaks in the *y* axes indicate thresholds of detection. Asterisks indicate statistical significance.

mock-treated mice this was already obvious by day 2. Initial studies on groups of three mice taken at days 3 and 7 after influenza virus infection revealed little difference in virus clearance. At day 3 the levels of PFU in the lung homogenates were $4.1 \times 10^4 \pm 1.8 \times 10^4$ for the mock-treated group and $2.7 \times 10^4 \pm 1.0 \times 10^4$ for the anti-IFN-γ-treated group. Both groups had cleared the virus by day 7 after infection. This is in agreement with a study of influenza virus infection in IFN-γ gene-targeted mice (10), which in a detailed kinetic study did not find any differences in virus clearance from wild-type mice. The preparation of lung homogenates for the determination of virus titers in lung tissue precludes the use of these mice for the study of immune responses in the lung parenchyma, and virus titers could therefore not be analyzed for the same mice.

Sera from all three experiments and lavage samples from one experiment were tested for their ability to inhibit the effects of rIFN-γ on the cell line WEHI-279. All sera and lavage samples from individual anti-IFN-γ-treated mice but not from the controls were able to neutralize rIFN-γ in this bioassay (Fig. 1), indicating that the MAb was administered in high enough amounts to reach the lungs as well as the circulatory system.

Effect of anti-IFN-γ treatment on anti-influenza virus Ig levels. The levels of influenza virus-specific IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA in serum, determined by ELISA for individual mice, are shown in Fig. 2 (note the logarithmic scale). Anti-IFN-γ treatment led to a >10-fold decrease ($P < 0.005$) in average levels of influenza virus-specific IgG2a at days 6 and 10 after infection and for IgG3 ($P < 0.02$) at day 6 after infection. The mean levels of virus-specific IgG3 at day 10 after infection were also modestly reduced (threefold). The levels of IgG1, IgM, and IgA in serum were not affected.

Effects of anti-IFN-γ treatment on cellular response in the respiratory tract. To investigate the effects of anti-IFN-γ treatment on the total and relative cell numbers within the respiratory tract, we performed FACScan analysis of MLN and lung parenchymal cells. Neither cell yield (Fig. 3) nor the proportion of cells in different cell populations within the MLN (Fig. 4) was significantly altered by the treatment. In contrast, in three independent experiments the total number of cells isolated from the lung parenchymas of anti-IFN-γ-treated mice was significantly reduced compared with that of mock-treated animals (Fig. 3). The first peak in the total number of cells, observed at day 2, was due to an increase in cells that appeared to be granulocytes, on the basis of their high side scatter, intermediate forward light scatter, and staining with a MAb against the granulocyte lineage marker GR1 (data not shown). At this time there was also an increase in cells with high forward light and high side scatter, but, as many of these cells were autofluorescent, they were gated out before FACScan analysis; these cells decreased in number and were much less prominent from day 4 after infection. The second peak, after day 4, was due to increases in the number of MAC-1^+ cells and $CD3^+/CD8^+$ T cells (Fig. 4).

FACScan analysis did not reveal a reduction or absence of any particular population and showed only some minor differences in the proportions of various cell types found in the lung parenchymas of influenza virus-infected mice treated with MAb against IFN-γ (Fig. 4). Lungs from both groups showed preferential increases in the numbers of $CD3^+$ and $CD8^+$ T cells from day 4 after infection.

Effects of anti-IFN-γ treatment on surface expression of activation and homing markers by $CD4^+$ and $CD8^+$ T cells from the lung parenchyma. The kinetics of expression from activation and homing markers on $CD4^+$ and $CD8^+$ T cells in the lung parenchymas of influenza virus-infected and anti-IFN-γ MAb-treated or mock-treated animals was studied next to determine whether anti-IFN-γ treatment had any effects on the surface expression of markers likely to be involved in lymphocyte adherence and migration. Figure 5 shows that the

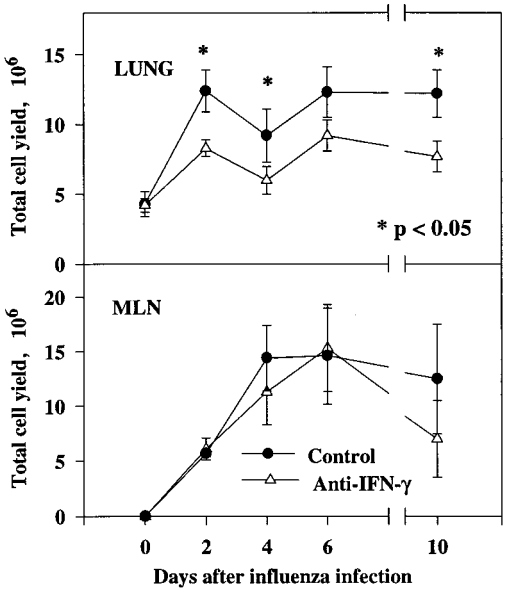

FIG. 3. Effects of anti-IFN-γ treatment on cellular infiltrate of lung parenchyma or MLN. The total cell yields obtained from lung parenchyma and MLN after infection of mice with Mem71 and treatment with rat anti-IFN-γ MAb or rat Ig (control) are compared. Shown are the means ± standard deviations (error bars) from three independent experiments. Asterisks indicate statistical significance.

surface expression of CD44 and CD62L, and to a lesser extent that of CD45RB and CD49d, by $CD4^+$ and $CD8^+$ T cells increased over the course of infection. Little difference between $CD4^+$ T cells isolated from mock-treated and anti-IFN-γ treated animals was found in the surface expression of these markers. $CD8^+$ T cells from the lung parenchymas of anti-IFN-γ treated animals, however, showed consistently from day 2 to 10 for CD62L, and from day 4 to 10 for CD49d, an increase in the frequency of cells expressing the activated forms of these markers ($CD49^{high}$ and $CD62L^{low}$, respectively).

Cytokine production in vitro. As shown in Table 1, cytokine production by unseparated cells isolated from the MLN and lung parenchymas of mice 7 days after influenza virus infection and then restimulated with anti-CD3 MAb in vitro was not affected by treatment of mice with anti-IFN-γ. Studies on cells separated into $CD3^+$ and $CD3^-$ cells before culture revealed no detectable IL-2, IL-3, IL-4, or IFN-γ production by $CD3^-$ cells, whereas supernatants of $CD3^+$ cells contained IL-2, IL-3, and IFN-γ activity at levels somewhat lower than those of unseparated controls (data not shown). This was also found when restimulation was done with virus-pulsed irradiated splenic antigen-presenting cells, suggesting that most if not all of the cytokine production in vitro was T cell derived. As described elsewhere (2), in vitro IFN-γ production by lung parenchyma T cells following influenza virus infection after anti-CD3 stimulation was 10- to 50-fold increased compared with that of T cells from noninfected mice. When tested here, the anti-IFN-γ-treated animals showed a similar increase in IFN-γ production by lung parenchyma cells, and the levels of IL-2 and IL-5 production were also not affected. IL-4 production by lung parenchyma cells from anti-IFN-γ-treated animals was detectable in the employed bioassay, whereas that of the infected mock-treated animals was always below the threshold of detection. While it was therefore difficult to assess the extent of the increase in IL-4 production in this system, the detected IL-4 levels were still very low and the unchanged levels of IgG1

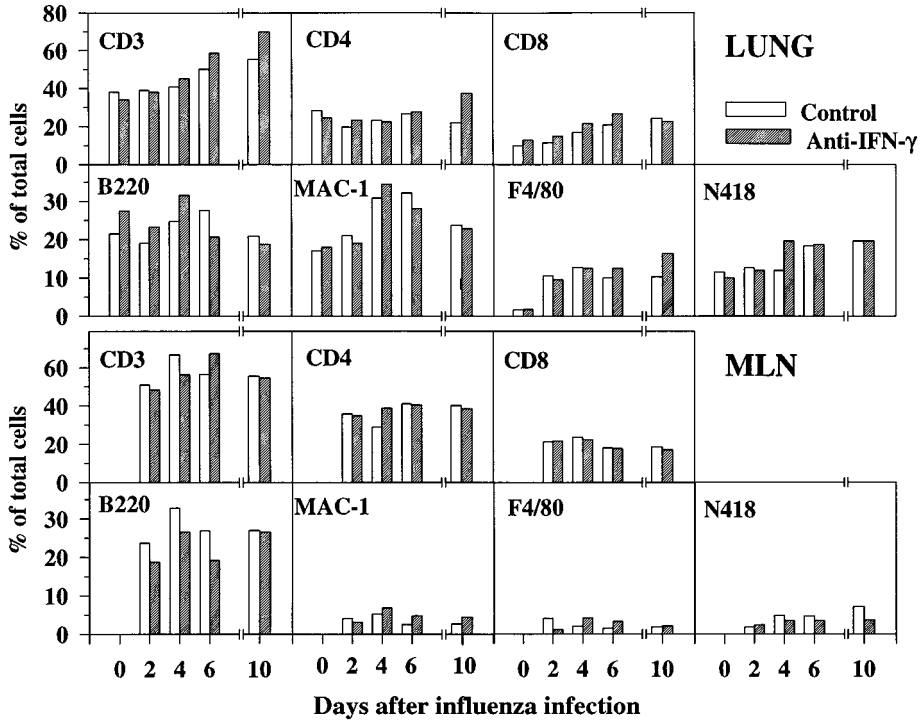

FIG. 4. FACScan analysis of cell suspensions from lung parenchymas and MLN taken from mice at indicated times after influenza virus infection. Mice received intraperitoneally a total of 3 mg of either rat Ig (control) or the anti-IFN-γ MAb XMG 1.2 (anti-IFN-γ). Shown are the means of two independent experiments.

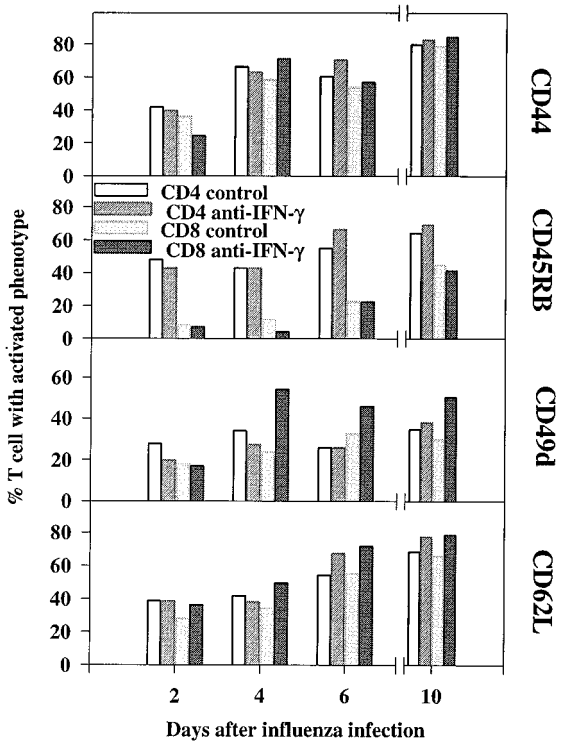

FIG. 5. Surface expression of activation and homing markers on $CD4^+$ and $CD8^+$ T cells in the lung parenchyma of mock-treated and anti-IFN-γ-treated mice at indicated times after infection with influenza virus. Shown are the relative number of T cells expressing the activated phenotype ($CD44^{high}$, $CD45RB^{low}$, $CD49d^{high}$, and $CD62L^{low}$) as obtained by FACScan analysis after logical gates were set for live cells by using propidium iodide exclusion and forward scatter-side scatter profile. Standard deviations for the percentage of activation marker-expressing cells were <4.5%, on the basis of data obtained in three independent experiments at day 7 after infection.

antibodies to influenza virus in vivo were also consistent with an unaltered T-cell cytokine profile.

To assess whether the kinetics of the influenza virus-induced cytokine response were altered by the neutralization of IFN-γ, graded numbers of cells from the lung parenchymas taken from mice at days 0, 2, 4, 6, and 10 after infection were cultured

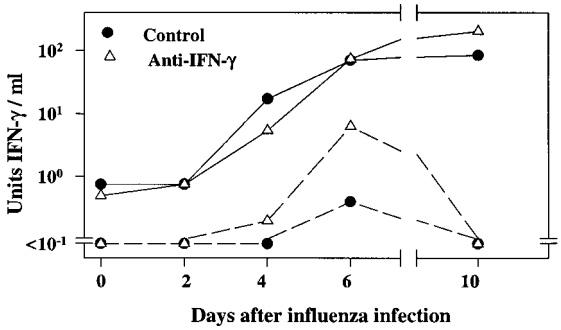

FIG. 6. Virus-induced cytokine production by lung parenchyma cells, from mock-treated (control) or anti-IFN-γ-treated mice, taken at indicated times after infection with influenza virus. Graded numbers of effector cells were cultured for 24 h with irradiated splenic antigen-presenting cells pulsed with influenza virus (solid lines) or with allantoic fluid of noninfected hen eggs (dashed lines). Shown are the mean values of IFN-γ activity determined from the supernatants of duplicate cultures containing 10^6 effector cells per ml.

in the presence of virus-pulsed or mock-infected irradiated splenic antigen-presenting cells. We observed a linear relationship between the number of effector cells added to the cultures and the cytokines measured (data not shown). Figure 6 depicts the results for IFN-γ production obtained with effector cells cultured at 10^6 cells per ml. Interestingly, only slight differences in the kinetics of IFN-γ production were obvious. At day 4 after infection there was a threefold reduction in the amounts of IFN-γ produced by lung parenchyma cells from anti-IFN-γ-treated animals, but by day 10 IFN-γ production by these cells was increased threefold compared with values for the control group. IL-4 was not detected in any of the culture supernatants tested. It appears therefore that short-term anti-IFN-γ treatment had only some minor effects on the kinetics of the virus-specific IFN-γ response, delaying cytokine production slightly; however, at the height of the response around day 6 to 7 there was little difference measurable. At this time after infection, cultures with mock-treated splenic feeder cells from both groups showed some low levels of IFN-γ production, which might be due to cells sufficiently activated in vivo to produce cytokines in this short-term assay. This spontaneous production of IFN-γ was sixfold increased in the cultures of lung tissue cells from anti-IFN-γ-treated mice.

TABLE 1. In vitro cytokine production by cells from the respiratory tracts of mice 7 days after influenza virus infection[a]

Cell source	Anti-CD3 MAb	Cytokine titer (U/ml) on cell line:			
		CTLL (IL-2)	CT.4S (IL-4)	T88-M (IL-5)	WEHI-279 (IFN-γ)
MLN					
Control	+	0.6	<0.01	0.2	1.9
	−	<0.01	<0.01	<0.01	0.3
Rat Ig and influenza virus	+	3.0	0.04	0.04	18
	−	<0.01	<0.01	<0.01	0.3
Anti-IFN-γ and influenza virus	+	3.0	<0.01	0.08	41
	−	<0.01	<0.01	<0.01	<0.04
Lung tissue					
Control	+	3.6	0.04	1.3	28
	−	<0.01	0.02	0.1	0.8
Rat Ig and influenza virus	+	1.2	<0.01	0.6	270
	−	<0.01	<0.01	0.05	0.1
Anti-IFN-γ and influenza virus	+	3.5	0.1	0.1	270
	−	<0.01	<0.01	<0.01	<0.01

[a] MLN and lung tissue cells from individual mice treated i.v. with rat Ig and infected i.n. with allantoic fluid (control), treated i.v. with rat Ig and infected i.n. with Mem71 (Rat Ig-influenza), and treated i.v. with anti-IFN-γ and infected i.n. with influenza virus (anti-IFN-γ–influenza) were cultured for 24 h with (+) or without (−) plate-bound anti-CD3 MAb.

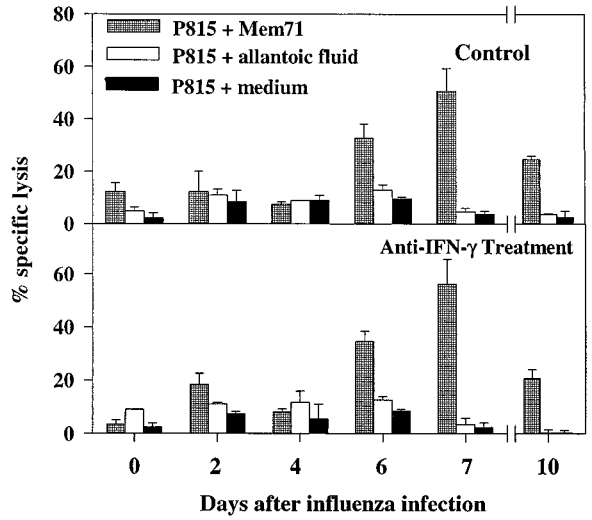

FIG. 7. Kinetics of ex vivo CTL activity in lung parenchyma. Results from one of two independent time course experiments which gave similar results are shown. ^{51}Cr release assays were performed at the indicated times after influenza virus infection and treatment with anti-IFN-γ MAb or rat Ig. Bulk populations of cells from lung parenchyma were cultured immediately ex vivo with ^{51}Cr-labelled P815 cells that were either infected with influenza virus or incubated with allantoic fluid or medium only, at an effector-to-target cell ratio of 100:1. Error bars, standard deviations.

Anti-IFN-γ MAb treatment did not influence effector CTL in lung parenchyma. We compared the CTL effector activities in the lung parenchymas of mice at various times after influenza virus infection and anti-IFN-γ treatment with those of infected mice that had received mock treatment only. Preliminary experiments showed that CTL could be detected directly ex vivo in lung tissue at the peak of the respiratory tract immune response and that this activity was eliminated if lung tissue cells were depleted of $CD8^+$ T cells. Figure 7 shows that neither the time of onset, the duration, nor the magnitude of $CD8^+$ T-cell-mediated killing per effector cell was altered by the antibody treatment, although anti-IFN-γ antibody activity was detected in the lavage samples of these mice (Fig. 1). This suggests that in vivo effector CTL activity in the respiratory tract did not depend on the presence of IFN-γ. As shown in Fig. 3, however, the total number of effector $CD8^+$ T cells in anti-IFN-γ-treated animals was reduced, indicating that the total level of CTL activity was lower in the lungs of anti-IFN-γ-treated mice.

In summary, the in vivo blockade of IFN-γ had measurable effects, firstly, on the systemic humoral immune response, by significantly reducing the levels of virus-specific IgG2a and IgG3 (Fig. 2), and secondly, on the cellular immune response in the lung parenchyma, a site in which influenza virus infection induces a large increase in the levels of IFN-γ mRNA (2). Surprisingly, the observed effect on the cellular response in the lung parenchyma was the alteration of leucocyte accumulation at this site (Fig. 3) and not a change in the ability of local T cells to produce cytokines (Table 1 and Fig. 6) and to exhibit CTL effector activity (Fig. 7). Consistent with the relatively low levels of IFN-γ mRNA found for the MLN (2), the in vivo blockade of IFN-γ did not reveal measurable effects on the cellular response at that site (Table 1 and Fig. 3 and 4).

DISCUSSION

Influenza virus infection causes striking local changes in the respiratory tracts of mice. As described previously (1, 2) this infection leads to marked increases in the number of cells recovered from MLN and lung tissue and a preferential accumulation of $CD8^+$ T cells in the lung parenchyma. We report here that this overall increase in the total number of cells found in the lung parenchyma but neither the preferential accumulation of $CD8^+$ T cells nor their cytolytic effector function was affected when IFN-γ activity was neutralized in vivo. Together the data suggest that during acute influenza virus infection, IFN-γ acts as a typical inflammatory cytokine which fine-tunes the local cellular immune response by regulating cell migration to the effector site. At the same time, this cytokine influences the type of the systemic humoral immune response by selectively enhancing the levels of virus-specific IgG2a, with its superior abilities to bind complement (23), to promote natural killer cell-mediated lysis of target cells (16), and to bind to high-affinity Fc receptors on macrophages (38).

Many studies have shown the induction of IFN-γ following influenza virus infection in different compartments of the respiratory tract: (i) in the draining MLN and cervical lymph nodes after restimulation in vitro (2, 4, 29) and at the mRNA level in $CD4^+$ and $CD8^+$ T cells (2, 4); (ii) in the lung parenchyma, where mRNA for IFN-γ was detected ex vivo in T cells and non-T cells (IFN-γ protein was detected in supernatants of restimulated cultures of these cells) (2); and (iii) in the airways, at the protein level in the BAL fluid (13, 35), and at the mRNA level in T cells and non-T cells (2a, 4, 28). Comparative analysis of the levels of IFN-γ production in vitro demonstrated >10-fold-higher levels of IFN-γ production by cells isolated from the lung parenchyma than by those from the MLN (2), which correlated closely with the >10-fold-higher levels of mRNA detected in lung parenchymal T cells than in MLN found in other experiments (2a). The fact that IFN-γ is upregulated in different tissue sites following influenza virus infection suggests that this cytokine is involved in particular aspects of the antiviral immune response in each compartment, in agreement with its reported pleiotropic effects. This study aimed to address in particular the question as to what the effect of IFN-γ might be in two of these respiratory tract compartments, namely the MLN and the lung parenchyma.

With regard to the cellular response in MLN, the short-term in vivo blockade of IFN-γ during influenza virus infection had little effect in this study, which is in agreement with a report in which a similar approach with β_2-microglobulin gene-targeted mice was used (30). The total number of cells and the cell composition in MLN were not affected, and cells isolated from this site produced levels of cytokines in culture comparable to those from mock-treated control mice. As the draining lymph nodes are generally regarded as the site of T-cell priming and the cytokine milieu of the site is believed to influence the cytokine pattern subsequently displayed by the induced effector T cells, the finding that short-term depletion of IFN-γ had no effect on the cytokine profile of the T cells is of importance. Our data might imply that, in this infectious model and in contrast to infection with *Leishmania major* (40), the development of a T cell into an IFN-γ producer does not require external IFN-γ and that other factors skew the response towards the induction of IFN-γ-producing T cells. Unaltered cytokine profiles were also reported for anti-IFN-γ-treated and influenza virus-infected β_2-microglobulin-deficient mice (30) and contrast with data on IFN-γ gene-targeted mice, for which higher levels of in vitro production of T-cell-derived IL-4 were found (10). While the reasons for this discrepancy are not clear, it could reflect the effects of the long-term absence of IFN-γ on the skewing of the cytokine profile toward IL-4 production by T cells in the latter mice, a notion that is supported by their increased levels of influenza virus-specific IgG1.

With regard to the humoral response and in agreement with many other studies, including a study on influenza virus-in-

fected and IFN-γ gene-targeted mice (10), we show here that the levels of virus-specific IgG2a in serum were significantly reduced. Interestingly, and in contrast to the study by Sarawar et al. (30), which detected a reduction in the number of virus-specific IgG2a- but not IgG3-producing cells in the MLN, we also noted a significant reduction in the levels of IgG3 in serum. The discrepancy between these two studies might be explained by the fact that the serum antibody response reflects responses not only in the MLN but also in the cervical lymph nodes, the lung parenchyma, and the spleen, where the effects of IFN-γ blockade may have been more marked. Antibodies of the IgG3 isotype are induced mainly against T-cell-independent antigens such as bacterial carbohydrate cell wall structures (11). There are discrepancies in the literature about the role of IFN-γ in regulating IgG3 production (14, 22, 32), and our findings are in agreement with a report showing a slight reduction in the total levels of IgG3 in mice lacking the IFN-γ receptor (14). This finding raises the possibility that whilst IgG3 production might not require cognate T-cell–B-cell interaction, IFN-γ production by T cells or non-T cells can help to increase the levels of IgG3 produced, a notion supported by earlier observations (22).

No studies on the function of IFN-γ in the lung parenchyma during influenza virus infection have been reported to date. As influenza virus replication is limited to the epithelial layers of the respiratory tract, the lung parenchyma together with the airways constitute the effector sites during the infection. Surprisingly, this study showed that the blockade of IFN-γ led to a reduction in the accumulation of leucocytes in the lung tissue. Lymphocyte recruitment into extralymphoid (tertiary) tissue sites is thought to be mediated through the expression of tissue-specific homing receptors on lymphocytes, induced after primary activation in secondary lymphoid organs such as draining lymph nodes or the spleen (25, 33). While the origin of lung parenchyma T cells is not known, that the accumulation of T cells occurs in MLN earlier than it does in lung tissue is consistent with their origin in the draining lymph nodes (Fig. 3 and 4).

Specific homing receptors that mediate recruitment of cells into the respiratory tract have not been described. Hamann et al. (12), however, found partial inhibition of the migration of blast cells into the lungs of normal mice after anti-lymphocyte function-associated molecule 1 (LFA-1) MAb treatment, consistent with the high levels of expression of LFA-1 that we observed on cells isolated from the lung parenchymas of both healthy and influenza virus-infected mice (2a). One of the ligands for LFA-1 is intercellular adhesion molecule 1 (ICAM-1), a molecule found on HEV, highly differentiated structures involved in the transmigration of lymphocytes from the bloodstream into the parenchyma of lymphoid organs. In the lung, HEV have been described in the bronchus-associated lymphoid tissue, lymphocytic aggregates in the lung found in most species (39), but also within lung parenchyma undergoing an immune response (reference 3 and references therein). Interestingly, IFN-γ, the cytokine which we have previously shown to be highly expressed in the respiratory tract as a result of influenza virus infection, has profound effects on HEV (3, 9, 15, 19, 26, 42), which suggests a regulatory role for IFN-γ in the events leading to the migration of leucocytes into secondary and tertiary tissue sites. With this study we extend these findings by providing, to our knowledge, the first in vivo evidence for a role of IFN-γ in the regulation of leucocyte migration into inflamed lung parenchyma, by demonstrating that anti-IFN-γ treatment led to a reduction in the total number of cells retained in the lung parenchyma at different times after influenza virus infection. Moreover, we demonstrate here that the in vivo blockade of IFN-γ increased the number of $CD8^+$ T cells expressing high levels of the α4-integrin chain (CD49d) and little CD62L. This raises the possibility that in the absence of IFN-γ alternative mechanisms of T-cell recruitment are employed, one of which might be the binding of α4-integrins to one of its ligands, leading to the accumulation of lymphocytes expressing this homing marker. This would also explain why anti-IFN-γ treatment resulted in only partial inhibition of leucocyte migration and why virus was cleared from the lungs at what appeared to be an unaltered rate. It seems that similar strategies for recruitment and/or retention of both lymphocytes and monocytes to this site have been utilized, as anti-IFN-γ treatment only marginally affected the cell composition of the lung parenchyma. It raises the possibility that local IFN-γ production affects the development of HEV in inflamed lung tissue and therefore the magnitude of leucocyte migration into the site. Histological examination of the tissues should allow this hypothesis to be tested.

Since we found a decrease in the total number of cells at the site in anti-IFN-γ-treated mice relative to that in control mice as early as day 2 after infection, IFN-γ production would have to be induced rapidly to influence this influx of cells. Indeed, Hennet et al. (13) were able to measure IFN-γ activity by bioassay in the lavage fluid of influenza virus-infected mice from 36 h after infection, and we found that $CD3^-$ cells in the lung parenchyma contained high levels of mRNA for this cytokine (2). Both non-T-cell populations known to produce IFN-γ, NK cells (36) and macrophages (8), are found in this tissue site, and NK cells have also been shown to be induced rapidly after influenza virus infection (34).

Anti-IFN-γ treatment of β_2-microglobulin gene-targeted and influenza virus-infected mice did not result in a reduction in the total number of cells accumulated in the airways but rather resulted in a selective reduction in the number of $\alpha\beta^+$ and $CD4^+$ T cells (30). The cell type that must have increased to compensate for the relative loss of $CD4^+$ T cells was not identified. As it is difficult in that system to dissect the individual effects the absence of class I molecules and IFN-γ might have on the regulation of the cellular response to influenza virus infection, a direct comparison between these two studies is difficult. Little is known about the function and origin of cells found in the BAL fluid and the relationship to cells found in the lung parenchyma (2a, 24). Both studies do, however, suggest that IFN-γ has selective effects in vivo on the recruitment of cells into different tissue sites.

Somewhat surprisingly, the treatment of mice with anti-IFN-γ MAb had no effect on the kinetics and the magnitude of the effector CTL responses in the lung, demonstrating that IFN-γ is not necessary for the generation of effector CTL in vivo and contrasting with earlier in vitro studies which showed an IFN-γ-dependent development of precursor CTL into effectors (20, 31). Our data also extend a study by Graham et al. (10) on influenza virus-infected mice with a disruption in the IFN-γ gene. That group was unable to demonstrate ex vivo killing when cells taken from spleens were tested but reported unaltered frequencies of precursor CTL at this site. Here we show that IFN-γ was also not required for the terminal differentiation of CTL in vivo. Notably, the data presented here clearly demonstrate how tightly effector CTL are regulated during influenza infection; not only were effector CTL confined to the lung parenchyma and airways and not detectable in MLN or the spleen (1, 2b, 10), but also ex vivo CTL activity was restricted to the period from about day 6 to 10 (Fig. 6), the time during which replicative virus is cleared from the lungs (3a). The differentiation of precursor CTL into effector CTL might therefore occur locally at the tissue site. The local action

of IFN-γ, however, does not seem necessary for either the differentiation or the observed rapid decline of CTL activity.

In summary, although IFN-γ appears not to be essential for recovery from a sublethal dose of influenza virus, this study has shown that it exerts a marked effect both on the humoral response and on the local immune response in the lung parenchyma where it can be found in very high levels after infection (2). We therefore propose that one of the functions of IFN-γ through which it exhibits antiviral activity is the promotion of optimal effector leucocyte migration and/or recruitment into the inflamed tissue site.

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