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Interferon Production by Leukocytes Infiltrating the Lungs of Mice During Primary Influenza Virus Infection

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Lung fluids and leukocytes were obtained from unprimed C3H mice by transpleural lavage at intervals after infection with influenza A/Hong Kong/68 virus and were tested for interferon activity. Lavage fluid interferon titers correlated directly with lung virus titers and with initial increases in leukocyte yields from infected lungs. In contrast to cultured lymph node cells from infected animals or leukocytes from lungs of uninfected mice, washed leukocytes obtained from the lungs of mice infected 2 to 6 days earlier produced interferon spontaneously in culture. The physiochemical, biological, and antigenic properties of both the interferon in lavage fluids and that produced by lung lavage leukocytes were similar and characteristic of alpha interferon. Fractionation studies indicated that macrophages and T lymphocytes were primarily responsible for the interferon produced in culture. The early presence and significant numbers of interferon-producing leukocytes in infected lungs suggests that these cells have an early role in defense against influenza virus infection.

Interferons are considered to be important in recovery from influenza virus infection, particularly during primary infection in which specific immune responses require time to develop (2, 17). Much of the evidence for this view is based on the close correlation observed between interferon levels and virus titers in secretions and lung fluids of humans and mice infected experimentally or naturally with influenza viruses (5, 17, 45; see 2 and 16 for reviews). More substantive evidence for a role for interferons in recovery from influenza disease is provided by studies which show the development of more severe influenza disease in T-cell-deprived mice unable to produce interferon (17) and in Mx mice treated with anti-mouse interferon globulin (11) than in appropriate control mice.

The cellular origins and the effects of interferons produced locally in influenza virus-infected lungs remain unclear. It has been suggested that interferons may affect local disease directly by inhibiting virus growth or indirectly by modulating phagocytosis (15) and diverse immune functions (24, 34, 35, 39). The type and degree of effect can vary according to the type of interferon induced, and the latter may differ under different conditions (39). For these reasons, the nature and origins of interferons must be determined separately for each virus-interferon model (34).

Studies of the cellular sources of the interferon induced during primary influenza virus infection have not produced a consensus. Macrophages and not lymphocytes from human peripheral blood have been reported as the main producers of interferon when these cells are exposed to influenza virus in culture (31). However, T lymphocytes and not adherent cells were found to be the primary producers of interferon when cultured splenocytes from uninfected mice were exposed to influenza virus (38). Similarly, irradiated mice, which are primarily deficient in lymphocytes, have been reported to be able (12) and unable (17, 25) to produce lung interferon during influenza virus infection.

In previous studies we observed infiltration of large numbers of leukocytes into the lungs of influenza virus-infected mice (42), and we used a transpleural lavage technique to obtain significant numbers of these cells for identification and study (41, 43). In this report, we focus on the relationship of these infiltrating leukocytes to local interferon production before detectable virus-specific immunity appears. The major leukocyte subpopulations which produce interferon in the lungs of unprimed mice infected with influenza virus are identified.

MATERIALS AND METHODS

Mice. C3H mice 8 to 12 weeks old, obtained from J. J. Trentin, Department of Experimental Biology, Baylor College of Medicine, Houston, Tex., were used in all experiments. All mice were housed in cages covered with barrier filters and were fed mouse chow and water ad libitum.

Virus. The isolation, preparation, and characterization of the ninth passage mouse-adapted influenza A/Hong Kong/68 (H3N2) virus used in these experiments has been described previously (42). Stocks of this virus in Hanks balanced salt solution containing 2% gelatin were filtered through a 0.45-μm filter (Acrodisc catalog no. 4181, Gelman Sciences, Inc., Ann Arbor, Mich.) and were stored in aliquots at −70°C. Approximately 100 PFU of this virus was equivalent to one median lethal dose (LD_{50}). A dose of 10 LD_{50} of this virus was given intranasally in a 0.05-ml volume to mice lightly anesthetized with ether in all experiments. Madin Darby canine kidney (MDCK) tissue cells were used to determine titers and to detect the presence of this virus (6).

Preparation of lung, spleen, and lymph node cell suspensions. Mice were killed by cervical dislocation. Their lungs were removed intact with the thoracic trachea, trimmed of detectable lymph nodes, rinsed in sterile saline, and lavaged transpleurally as described previously (43). Lung lavage cells (LC) from 6 to 10 mice were pooled and centrifuged for 10 min at 600 × *g*. Supernatant lavage fluids were passed through a 0.45-μm filter and were frozen in aliquots at −70°C until assayed for interferon levels. The pelleted cells were depleted of erythrocytes by lysis in isotonic Tris-buffered ammonium chloride solution (pH 7.3), washed two times, and suspended to 10^6 cells per ml in Eagle minimal essential medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% fetal calf serum.

Spleens and mediastinal lymph nodes were teased into suspension and washed through a 60-mesh stainless steel screen with sterile saline. The cells were pelleted by centrifugation, depleted of erythrocytes, washed two times, and suspended in minimal essential medium. All cells were maintained on ice until processed or added to culture plates.

Fractionation and depletion of leukocyte subpopulations. Suspensions enriched for nonadherent or adherent leukocytes were prepared by allowing adherent cells to selectively adhere to plastic petri plates coated with fetal calf serum as described by Kumagai et al. (22). Two sequential incubations on the plastic plates were done to ensure optimum fractionation in each experiment.

Specific leukocyte subpopulations were depleted with specific antisera and complement in a two-step procedure. In step 1, the adherent or nonadherent cell fractions obtained after incubation on plastic were placed on ice and incubated with dilutions of antisera previously determined to be optimum for depletion. After 60 min, these cells were washed and incubated at 37°C in the presence of rabbit Lo Tox complement (catalog no. CL3051; Cedarlane Laboratories, Hornby, Ontario, Canada). To deplete macrophages, fractions were incubated with rat mon-monoclonal immunoglobulin G antibody against an alloantigen unique to mouse macrophages (33; catalog no. MAS0346, Sera Lab, distributed by Accurate Chemical Co., Westbury, N.Y.). T lymphocytes were depleted from suspensions with either absorbed mouse anti-theta serum (catalog no. 8301-01, Litton Bionetics, Kensington, Md.) or a combination of monoclonal antibodies with specific activities for the LyT-1.1 or LyT-2.1 alloantigens of T lymphocytes (catalog no. CL-8911 and CL-8921, respectively, Cedarlane Laboratories). Natural killer cells were depleted with monoclonal antibody to mouse Ly-5.1 alloantigen (catalog no. NEI 020, New England Nuclear Corp., Boston, Mass.). Goat antiserum with specificities for mouse immunoglobulins (catalog no. 0111-0231, Cappel Laboratories, Cochranville, Pa.) was used to deplete B lymphocytes. The specificities and use of these antisera to identify or deplete specific subpopulations have been described elsewhere (4, 13, 18, 29, 33).

Cell quantitation and identification. Tissue culture cells and nucleated cells in leukocyte suspensions were counted with a standard hemocytometer. Cell viability was determined by trypan blue exclusion. Differential counts were made on cells spun onto slides with a cytocentrifuge and stained with a procedure to detect nonspecific esterase (36), or on cell suspensions stained with acridine orange (10).

Cells bearing surface membrane immunoglobulin (B cells) or Thy-1 antigen (T cells) were quantitatively assessed by direct immunofluorescence (29, 40) with fluorescein isothiocyanate-conjugated goat anti-mouse gamma globulin (Cappel Laboratories) or absorbed fluorescein isothiocyanate-conjugated rabbit anti-mouse brain (C3H) sera (catalog no. 8301-63, Litton Bionetics), respectively. All slides were examined at ×400 with a Leitz Orthoplan fluorescent microscope equipped with phase optics and Ploem illumination. Lymphocytes positive for staining were recognized by a rim of fluorescence encompassing the cell circumference.

Cell cultures. To measure cellular production of interferon, fractionated and unfractionated LC, lymph node cells, and splenocytes were washed three times, serially diluted in minimal essential medium (5% fetal calf serum), and added in 1-ml volumes to wells in 12-well culture plates (Costar 3512; diameter, 12 mm) in the absence of known virus. All plates were incubated at 37°C in 5% CO_2 for 96 h. At 24-h intervals, fluids from sets of replicate cultures were harvested, centrifuged, and filtered through 0.45-μm filters. These fluids were then frozen in aliquots at −70°C until assayed for interferon activity.

Assay of interferon. Interferon was assayed, its presence being shown by the inhibition of cytopathic effect (CPE) caused by vesicular stomatitis virus (VSV) in monolayers of L929 fibroblasts (34). Each assay was performed in duplicate and incorporated a mouse interferon standard preparation obtained from J. K. Dunnick and J. R. La Montagne of the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. Interferon titers were expressed as the reciprocal of the last dilution which completely inhibited CPE. The titer of the standard used in these assays rarely varied from assay to assay, and endpoints were usually equal to the stated titer of the reference interferon (1,200 U/ml).

The ability of test samples to inhibit growth of influenza viruses was determined in monolayers of primary mouse embryo (PME) cells. PME monolayers were exposed to the test samples for 16 h, washed, and then challenged with approximately 100 median tissue culture infective doses of influenza A/Hong Kong/68 or B/Hong Kong/72 viruses. After a 90-min adsorbtion period, the monolayers were rinsed, and minimal essential medium was added. After 3 days, each culture fluid was tested for virus hemagglutination

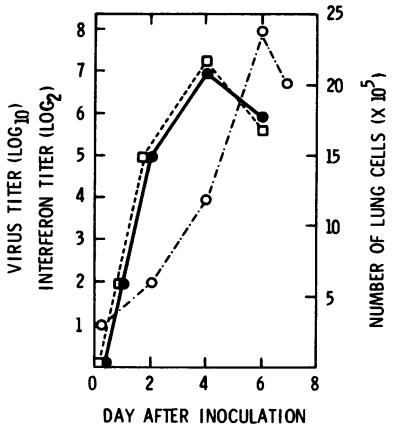

FIG. 1. Comparison of titers of lung interferon (●) and virus (□) with the number of lung lavage leukocytes (○) obtainable from the lungs of uninfected mice or mice infected intranasally with influenza A/Hong Kong/68 virus.

units. One hemagglutination unit was equal to the reciprocal of the last dilution of culture fluid which hemagglutinated a 1.0% suspension of chicken erythrocytes.

Characterization of interferon samples. Trypsin, pH, and heat stability were tested as previously described (31, 44). The ability of rabbit anti-L cell (type I) interferon globulin (Antiviral Substances Program, National Institute of Allergy and Infectious Diseases) to neutralize antiviral activity was tested by incubating equal volumes of test samples and dilutions of the anti-interferon globulin for 1 h at 37°C. Control samples were incubated with normal rabbit serum or medium. All samples were then assayed for antiviral activity as described above. In most tests a 10^{-3} dilution of globulin (stated titer, 6,000 U/ml) reduced antiviral activity >99%.

RESULTS

Effects of infection on local responses. Figure 1 compares the levels of influenza virus and interferon in the lungs of C3H mice infected intranasally with 10 LD_{50} of influenza virus with the number of leukocytes obtainable by transpleural lavage. Quantities of virus and interferon in infected lungs rose rapidly after virus inoculation, peaked on day 4, and then declined. No interferon or virus was detectable in the lungs of surviving mice on day 10 (data not shown). Increases in the number of LC lagged behind rises in virus and interferon titers and reached maximum levels on day 6.

LC obtained from both uninfected and infected lungs were 85 to 95% viable and consisted primarily of macrophages and lymphocytes in approximately equal proportions. T lymphocytes were the predominant lymphoid subpopulation, ranging from 25 to 36% of the total cells observed. B lymphocytes and null lymphocytes comprised from 6 to 12% and from 8 to 2% of the total cells in uninfected and infected lungs, respectively. Polymorphonuclear neutrophiles and nonleukocytes together seldom exceeded 10% of the total cells counted.

Protection of L929 cells from VSV-induced CPE. Medium from LC harvested by transpleural lavage 2 to 6 days after infection and cultured in the absence of L929 fibroblasts or added virus consistently protected L929 cells from CPE induced by VSV (Tables 1 and 2, line 1). Maximum titers of interferon were obtained with LC harvested from animals infected 4 days. Interferon levels in these cultures usually peaked 48 h after initiation of the cultures and varied in direct proportion to the number of LC cultured. These data are similar to those ob-

TABLE 1. Evidence that the interferon in lung lavage fluids from influenza virus-infected mice and in fluids from lung lavage leukocyte cultures is primarily type alpha[a]

Treatment[b]	Interferon in[c]:			
	Lung fluid		Culture fluid	
	Titer	P	Titer	P
None	6.0 ± 0.7		4.0 ± 0.3	
Heat (56°C, 60 min)	2.8 ± 1.3	<0.05	UD[d]	<0.05
pH 2	5.6 ± 0.5	NS[e]	3.7 ± 0.3	NS
Trypsin	UD	<0.01	UD	<0.05
Anti-mouse type I interferon	UD	<0.01	UD	<0.05
Anti-mouse gamma globulin	5.5 ± 0.4	NS	4.0 ± 0.2	NS

[a] Both the lung lavage fluids and leukocytes that were cultured were obtained by transpleural lavage of lungs harvested 4 days after infection with 10 LD_{50} of influenza A/Hong Kong/68 virus.

[b] See text for procedures and reagents used.

[c] Untreated and treated fluids were tested for inhibition of CPE by VSV in L929 cells. Titers shown are GMT $\log_2$ ± standard deviation; $n \geq 3$. Values were compared by Student's t test.

[d] UD, Undetectable.

[e] NS, Not significant.

TABLE 2. Determination of the major leukocyte subpopulations in lungs of unprimed C3H mice infected with influenza A/Hong Kong/68 virus that produced interferon spontaneously when placed into culture[a]

Lung lavage cell population cultured[b]	Interferon titer[c] (GMT $\log_2$ ± SD)
Unfractionated	4.3 ± 0.6
+ A-Mϕ + C	4.3 ± 0.6
+ A-Thy-1 + C	4.3 ± 0.6
+ A-LyT-1,2 + C	4.3 ± 0.6
With A-IF in medium	UD
Adherent	4.3 ± 0.6
+ A-Mϕ + C	UD
+ A-Thy-1 + C	4.0 ± 0
+ A-LyT-1,2 + C	4.0 ± 0
+ A-Ly-5 + C	4.0 ± 0
With A-IF in medium	0 ± 0
Nonadherent	4.3 ± 0.6
+ A-Mϕ + C	4.0 ± 0
+ A-Thy-1 + C	UD
+ A-LyT-1,2 + C	UD
+ A-Ly-5 + C	1.7 ± 0
+ A-Mo Ig + C	4.0 ± 0
With A-IF in medium	UD

[a] All leukocytes were obtained by transpleural lavage from mice inoculated 4 days previously with 10 LD_{50} of influenza A/Hong Kong/68 virus. LC from 6 to 10 mice were pooled for each experiment. Abbreviations: A, Anti; Mϕ, macrophage; C, complement; LyT-1,2, combined LyT-1 and LyT-2 antisera; IF, interferon type I (alpha); Mo, mouse; Ig, immunoglobulin; UD, undetectable.

[b] See text for procedures and reagents used.

[c] Unfractionated or fractionated lavage leukocytes (3×10^6 per well) were cultured for 72 h in 12-well tissue culture plates (Costar 3152). The culture fluids were then removed and tested for the maximum dilution which completely inhibited CPE induced by VSV in L929 cells. GMT $\log_2$ ± standard deviations of three replicate experiments are shown.

tained with the influenza virus-PME cell system (Fig. 2) and so are not shown. No detectable interferon was ever observed in medium from cultures containing LC obtained from uninfected mice or from cultures of LC obtained 10 days after infection.

Because the possibility existed that interferon might be induced in the L929 test cells by influenza virus carried over with or released by cultured LC from infected mice, medium from LC was assayed on MDCK cells and was found to be negative for detectable virus. Moreover, when infectious influenza virus was added to L929 cell cultures in quantities ranging from 10^2 to 10^6 PFU, no interference with the development of VSV-induced CPE was observed (data not shown).

Protection of PME cells from influenza virus. The ability of culture medium from incubated LC to inhibit the growth of influenza viruses was assessed by adding test samples to PME cultures 16 h before challenge with influenza A/Hong Kong/68 or B/Hong Kong/72 viruses and observing the inhibition of virus growth. Activity inhibitory to the growth of influenza viruses was present in media from cultured lung leukocytes obtained 4 days after infection (Fig. 2), and as in the VSV-L929 cell system, the activity was proportional to the number of leukocytes cultured. These data also indicate that influenza viruses homologous (A/Hong Kong/68) and heterologous (B/Hong Kong/72) to the virus used to infect the mice were both inhibited. Similar inhibition of the growth of influenza viruses in PME cells was observed when LC or lavage fluid from mice infected 4 days were added to wells, but no inhibition occurred when similar materials derived from uninfected mice or mice infected 10 days were tested (data not shown).

Characterization of the inhibitor and producing cells. Lavage and culture fluids that were inhibitory to virus growth on day 4 after infection retained this activity after dialysis against glycine-hydrochloric acid buffer, pH2, at 4°C for 24 h or after the addition of anti-mouse gamma globulin (Table 2). However, geometric mean titers (GMT) of both types of fluids were significantly reduced after 60 min of incubation at

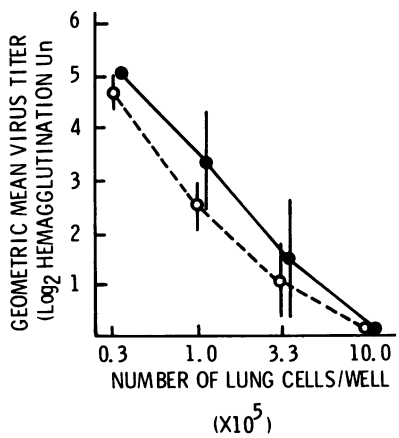

FIG. 2. Inhibition of influenza viruses in PME monolayers by medium from cultures of lung leukocytes obtained from mice infected with influenza A/Hong Kong/68 virus 4 days earlier and placed into culture in the absence of added virus. Shown is the proportional relationship of inhibitory activity to the number of leukocytes in each culture well. All fluids were removed 72 h after culture initiation and were tested for the inhibition of growth of influenza A/Hong Kong/68 virus (●) or influenza B/Hong Kong/72 virus (○). Virus titers are presented as hemagglutination units (GMT $\log_2$ ± standard deviation; $n = 3$).

56°C, and antiviral activity was obliterated by treatment with trypsin and by neutralization with anti-mouse type I interferon. In additional tests, HR218 human foreskin fibroblasts were not protected from VSV-induced CPE after exposure to the same lavage and culture fluids that were active on PME or L929 cells. This species restriction, together with the physiochemical and antiserum neutralization data, indicated that the antiviral factor present in both lung lavage fluids and in fluids from cultured LC was similar and was type alpha (type I) interferon (31, 44).

LC subpopulations producing interferon were determined by testing culture fluids from unfractionated or fractionated LC for antiviral activity. Test leukocytes were obtained from mice infected 4 days with influenza A/Hong Kong/68 virus and were incubated 72 h in 12-well tissue culture plates. All fractions were multiply washed before culturing and were incubated in the absence of added virus. The GMT $\log_2$ indicate that unfractionated, adherent, and nonadherent cells obtained from unprimed infected mice all produced equivalent levels of interferon in culture (Table 2). In contrast, no detectable interferon was observed in fluid from cultures to which anti-mouse interferon globulin had been added. None of the antisera to cell surface antigens inhibited interferon formation in unfractioned lung leukocytes. However, interferon formation by adherent cells was prevented by treatment with antimacrophage serum plus complement, and interferon formation by nonadherent cells was prevented by antisera to T lymphocytes (anti-theta, or anti-LyT-1 and anti-LyT-2 in combination) plus complement, but not vice versa. Interferon activity in culture fluids from both adherent and nonadherent cell subpopulations was inhibited by the anti-mouse type I interferon serum. Anti-Ly-5.1 serum plus complement had no effect on interferon production by adherent cells, but it reduced interferon production by nonadherent cells by about half. Anti-mouse immunoglobulin plus complement had no effect on interferon production by nonadherent cells. These data indicate that macrophages and T lymphocytes are the two predominant leukocyte subpopulations producing interferon in mouse lungs during primary influenza virus infection, and that both cell types are producing alpha (type I) interferon.

DISCUSSION

In the present study, production of interferon by LC from uninfected or influenza virus-infected mice was determined by adding fluids from cultured LC to monolayers of L929 or PME cells and then observing for inhibition of virus growth. In general, the higher the titer of interferon observed, the greater the concentration of interferon-producing cells present in the original lung suspension. The proportional increases in interferon levels with increased numbers of leukocytes cultured (Fig. 2) suggest that this premise is valid. Interferon-producing cells were first detected in lung lavage suspensions on day 2 after infection with mouse-adapted influenza virus, a time concomitant with the first detection of significant levels of interferon in lavage fluids, and reached maximum numbers on days 4 and 6, the days that maximum lung interferon titers were observed. Differential counts of cells in lung lavage suspensions indicated that 85 to 90% of the cells present were macrophages and lymphocytes with polymorphonuclear neutrophiles comprising most of the remaining cells. It is not known what proportion of the leukocytes immigrated into the lung from the peripheral blood or proliferated from indigenous lymphoid tissue. However, fractionation and deletion experiments to evaluate the relative contribution of these leukocyte subpopulations to the observed interferon levels suggested that T lymphocytes and macrophages, the two predominant types of leukocytes present in these suspensions, were the major subpopulations producing interferon and involved in protecting L929 and PME monolayers from infection with virus.

Because of the difficulty in separating or fractionating some leukocyte subpopulations, the possibility of interferon production by a small subpopulation of cells other than macrophages and T cells cannot be ruled out. Natural killer cells have been reported in influenza virus-infected lungs (23) and are known to produce interferon when exposed to virus (39). These cells have been found to have low levels of Thy-1 alloantigen on their cytoplasmic membrane (14), making them particularly difficult to separate from T lymphocytes and to identify. However, the protection of monolayers and production of interferon over a broad range of cell dilutions argues against a significant contribution by a numerically small cell population. Moreover, natural killer cells express Ly-5 but not Ly-1 or Ly-2 surface antigens (13, 14, 18, 20) and anti-Ly-5 sera and complement failed to eliminate the protective activity of either the adherent or nonadherent fraction in these experiments. The reduction of interferon titers observed in culture fluids from nonadherent cells treated with anti-Ly-5 sera and complement could be attributed to a reduction in T lymphocytes, which may also express Ly-5 surface markers (18, 20).

LC from influenza virus-infected mice appeared to be actively producing interferon when placed into culture. The cells were washed several times before being added to wells, and they were incubated in the absence of added virus, yet significant levels of interferon were observed

in culture fluids. Maximum interferon titers were usually reached at 48 h of culture. Production may have been terminated at this time because of decreases in the number of viable cells in culture or because of a lack of virus stimulation after culture. It is worth noting that interferon-producing cells were not detected in mediastinal lymph nodes from infected mice at any interval observed during these tests, suggesting that infectious virus or virus products were responsible for stimulating lung leukocytes to produce interferon.

Despite attempts to elucidate them, the cellular origins of the interferon produced locally in the lungs during virus infection of unprimed mice have not been clear. It has been noted that circulating interferon induced by myxoviruses and paramyxoviruses, in contrast to that induced by many other viruses, was produced by radiosensitive cells, most probably lymphocytes (7, 25). However, lung interferon levels in these irradiated mice were not decreased, suggesting that macrophages were involved in interferon production at the immediate site of infection. Similarly, it has been shown that mice depleted of T cells by irradiation and surgery, anti-lymphocyte serum treatment, or immunosuppressive hormone treatment produce no detectable circulating or lung interferon (3, 7, 17, 28). However, T-cell-deficient nude mice inoculated with Newcastle disease or lymphocytic choriomeningitis viruses, although initially deficient in interferon, have been shown to have levels of circulating interferon comparable with (27) or higher than (26) normal mice at ≥10 h after inoculation. Why nude mice should differ from other T-cell-deficient mice is unclear. However, these mice reportedly have some T-cell activity as well as compensatory increase in reticuloendothelial activity (27).

In vitro studies concerning the cellular sources of interferon have also produced varied results. The main interferon-producing cells in spleen cell cultures from normal mice exposed to herpes, vaccinia, or influenza viruses were observed to be T cells, with only limited production of interferon by macrophages (30, 37, 38). However, numerous studies have demonstrated the capacity of macrophages to produce interferon (1, 8, 9, 19, 21, 31, 32). In one of these studies (31), human monocyte-derived macrophages exposed to influenza virus in vitro produced interferon, but lymphocytes did not. The data presented in this report indicate that both macrophages and T lymphocytes are active in producing alpha interferon in lungs of unprimed mice infected with influenza virus.

Despite the marked dilution of respiratory secretions in the lung lavage procedure, significant levels of interferon were observed in these fluids. Thus, high titers of interferon must be present at the mucosal surfaces in infected lungs. The demonstration by others that T-cell-deficient mice unable to make interferon (17) and Mx mice treated with anti-mouse interferon globulin (11) had worse disease than did comparably inoculated control mice suggests an important role for this interferon. Moreover, virus-specific T lymphocytes, the first detectable virus-specific immune response, are detectable 3 to 4 days after primary influenza virus infection (41); alpha interferon and interferon-producing cells appear 1 to 3 days earlier and thus represent a very early defense mechanism in influenza disease.

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