Production of Interferon by Alveolar Macrophages

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ABSTRACT

ACTON, JEAN D. (Bowman Gray School of Medicine, Winston-Salem, N.C.), AND QUENTIN N. MYRVIK. Production of interferon by alveolar macrophages. *J. Bacteriol.* **91**:2300-2304. 1966.—Rabbit alveolar macrophages inoculated with para-influenza-3 virus in vitro produce a viral inhibitor which possesses the properties of interferon. The interferon is nondialyzable, is stable at pH 4, is not sedimented at 100,000 $\times g$, exhibits species specificity, and can passively protect other alveolar macrophages from infection with virulent rabbitpox virus. The possible significance of alveolar macrophage-produced interferon is discussed.

In spite of increased interest in the interaction of viruses with the cells of the reticuloendothelial system (RES), there exists a paucity of investigations on the function of alveolar macrophages in viral infections with respect either to their role in immunity to viral respiratory diseases or their role as host cells for propagation of virus.

Liu (12) was one of the first investigators to demonstrate viral antigens in alveolar macrophages. He employed fluorescent-antibody techniques to identify the viral antigens present within the cells in nasal smears and sections of lungs obtained from ferrets infected with influenza virus.

In a study on the histopathogenesis of mousepox virus in mice, Roberts (19) observed that alveolar macrophages were among the first cells to contain viral antigens after aerosol infections. He also demonstrated that reticuloendothelial cells of the liver were important in the uptake of viruses present in the vascular compartment.

Gresser (4) was probably the first investigator to report that human white blood cells from venous blood had the capacity to produce interferon. His findings suggested that cells of the RES might contribute to the host's defense mechanisms against viral infections by producing interferon.

Several investigators (1, 6, 7, 10, 17, 22, 23) have attempted to define the possible role of interferon in protecting against and in effecting recovery from viral infections. Collectively, their findings suggest that interferon may be an important factor of immunity during the early phases of viral infection before antibody immunity becomes expressed.

The present study was initiated to investigate

the possible production of interferon by virus-infected alveolar macrophages as well as the feasibility of passively immunizing macrophages in vitro with alveolar macrophage-produced interferon.

MATERIALS AND METHODS

Animals. New Zealand white rabbits weighing between 1.8 and 2.2 kg were utilized as the source of alveolar macrophages. They were killed by injecting air into the marginal ear veins.

Procurement of alveolar macrophages. Alveolar macrophages were obtained from rabbit lungs by a slight modification of the procedure described by Myrvik, Leake, and Fariss (16). The rabbit lungs were removed with the trachea clamped off with a hemostat. The cells were flushed from the lungs by injecting and withdrawing Hanks' balanced salt solution at a point below the hemostat.

Cell cultures. Alveolar macrophages were maintained as suspension cultures in Medium 199 in Earle's base (BBL) supplemented with 1.1 g of NaH_2PO_4 per liter and 20% heat-inactivated calf serum (56 C for 30 min). This "spinner modified" medium contained 100 units of penicillin and 100 μg of streptomycin per ml.

HEp-2 cells, obtained from William S. Jordan, Jr., University of Virginia School of Medicine, were grown as monolayers in Medium 199 supplemented with 10% heat-inactivated calf serum (Medium 199-10% Ca). No antibiotics were added to this growth medium.

All cells were maintained at pH 7.4 to 7.5 in humidified 5% CO_2 in air at 35 to 36 C.

Viruses. Rabbitpox virus (Utrecht strain), obtained from the American Type Culture Collection, was propagated in monolayers of HEp-2 cells maintained in Medium 199 supplemented with 5% heat-inactivated calf serum (Medium 199-5% Ca).

Parainfluenza-3 virus, obtained from Clayton Wheeler, University of North Carolina School of Medicine, was propagated in monolayers of HEp-2 cells maintained in Medium 199 supplemented with 5% heat-inactivated chicken serum (Medium 199-5% Ch).

Virus inocula were prepared by freezing and thawing infected cell suspensions three times. The virus-containing supernatant fluids were stored in 1-ml samples at -70°C .

Virus titrations. Rabbitpox virus was assayed in HEp-2 cell monolayers by a modification of the procedure described by Postlethwaite (18) for plaquing vaccinia virus without an agar overlay. The cells were grown for 24 hr in 60-mm plastic petri dishes (Falcon Plastic Co., Los Angeles, Calif.). The growth medium was removed, and 0.5 ml of inoculum, diluted in Joklik's (9) modification of Puck's saline A (13), was allowed to adsorb for 30 min at 35 to 36°C . A 4-ml amount of a fluid overlay (Medium 199-5% Ca) was added. After 18 to 24 hr of incubation, this medium was replaced with Medium 199 containing 2% heat-inactivated calf serum. At 36 to 48 hr after inoculation, the medium was removed and the plates were stained for approximately 30 sec with a 1% aqueous solution of crystal violet, washed with tap water, and dried. The technique of reducing the serum content after 18 to 24 hr of incubation reduced the growth rate of the cells, which maintained the cell sheet as a monolayer. The initial 5% concentration of serum was necessary to obtain optimal plaque development.

Parainfluenza-3 virus was assayed in tubes of HEp-2 cells by a modification of the hemadsorption technique (21). Half-log dilutions of the virus were made in Hanks' balanced salt solution (BSS), pH 7.4 to 7.6, and 0.2 ml of inoculum was added to each of two or three cell cultures. The virus was allowed to adsorb for 1 hr at 35 to 36°C , after which 1 ml of maintenance medium (Medium 199-5% Ch) was added to each tube. The culture fluids were changed after 24 hr of incubation. At 48 to 72 hr after inoculation, 0.2 ml of 0.4% guinea pig red blood cells in 0.14 M NaCl was added to each tube. The cultures were incubated at 4°C for 30 min, washed with 5 ml of Hanks' BSS, and observed microscopically for hemadsorption and cytopathogenic effect (CPE).

Estimation of viral CPE in alveolar macrophages. Viral cytopathogenic effect in alveolar macrophage preparations was estimated by determining the percentage of the cells which did not exclude trypan blue (14).

Interferon assay. For virus challenge, extracts of virus-infected and uninfected control fluids, which were to be assayed for the presence of viral inhibitor, were diluted with equal amounts of spinner modified medium. Alveolar macrophages were suspended in the diluted preparations at a cell concentration of 400,000 cells per milliliter. The spinner flasks were incubated at 35 to 36°C for 18 hr in a CO_2 incubator. After centrifugation at $200 \times g$ for 10 min, the cell pellets were resuspended in half the original volume of spinner medium. Rabbitpox virus inoculum was added to the cell suspension at a multiplicity of ap-

proximately 0.1 plaque-forming unit (PFU) per cell, and the cell concentration was adjusted to 400,000 cells per milliliter with additional spinner medium. The viral inoculum was allowed to adsorb for 3 hr in a CO_2 incubator. The cell suspension was then centrifuged at $200 \times g$ for 10 min, and the cell pellet was resuspended in spinner medium at the original cell concentration. Samples for viral titrations were removed at desired intervals and stored at -70°C . At each sampling interval, cell counts and the percentage of viable cells were determined. A virus control was incubated for 18 hr in fresh spinner medium rather than interferon or control fluid before inoculation with rabbitpox virus. An uninfected cell control was treated as above except that rabbitpox virus was omitted.

In the assay for plaque reduction, the samples removed at the various intervals were assayed for infectious virus in HEp-2 cell monolayers as described in Materials and Methods. A 75% reduction in the number of plaques in the test system, as compared with the number of plaques in the control system, was considered to be a significant difference.

RESULTS

Growth of rabbitpox virus in alveolar macrophages. Studies were carried out to determine the course of rabbitpox virus infection in rabbit alveolar macrophages in vitro. Suspension cultures of macrophages at a cell concentration of 400,000 cells per milliliter were infected with rabbitpox virus at a multiplicity of approximately 0.1 PFU per cell. Infected and uninfected cultures were incubated at 35 to 36°C in a CO_2 incubator for 1 hr. After centrifugation at $200 \times g$ for 10 min, the cells were resuspended in spinner modified medium. Samples were harvested periodically and were assayed for free and cell-associated infectious virus in HEp-2 cells as previously described. An increase in the quantity of cell associated virus was first observed between 9 and 12 hr after infection. An increase in the quantity of virus in the supernatant fluids was observed 12 to 18 hr after addition of virus. (The infected alveolar macrophages in spinner cultures yielded a higher percentage of virus to the supernatant fluid than did infected HEp-2 cell monolayers. This could be due either to the different pattern of CPE production in alveolar macrophages by rabbitpox virus or to mechanical disruption of the cells in the spinner flasks.) Approximately a 10% decrease in cell viability was observed at this interval. Maximal virus production (10^5 to 5×10^5 PFU per ml) was achieved 24 to 48 hr after infection.

Failure of alveolar macrophages to support the growth of parainfluenza-3 virus. Suspension cultures of macrophages at a cell concentration of 400,000 cells per milliliter were inoculated with

parainfluenza-3 virus to give a range of 0.05 to 1.0 TCID₅₀ per cell. The procedures for infecting and sampling were the same as those described for growth of rabbitpox virus. Samples obtained from the macrophage suspensions were titrated for the presence of parainfluenza-3 virus as described in Materials and Methods.

Multiplication of parainfluenza-3 virus in macrophages was not observed under the conditions of these experiments. The macrophage-associated virus, which was released by freezing and thawing the cells, decreased in parallel with the free virus in the supernatant fluid. No infectious virus could be recovered 6 to 9 hr after the addition of the viral inoculum to the macrophage cultures. Paradoxically, extensive CPE developed in the cultures even though the parainfluenza-3 virus disappeared and apparently failed to replicate.

In vitro interferon production by macrophages. Suspension cultures of macrophages were inoculated with parainfluenza-3 virus to give a range of 0.05 to 1.0 TCID₅₀ per cell. Samples were removed from virus-infected and uninfected control cultures at 12, 18, 24, 48, and 72 hr after inoculation. All preparations were frozen and thawed three times and centrifuged at $820 \times g$. The supernatant fluids were then centrifuged at $100,000 \times g$ for 1 hr at 4 C. These crude preparations were assayed for interferon activity as described in Materials and Methods.

Interferon-like activity was detected when either 0.1 or 0.25 TCID₅₀ was employed; however, 0.05 TCID₅₀ failed to induce measurable interferon. Inocula containing 1.0 TCID₅₀ resulted in early cell death and no interferon production. The results indicated that maximal interferon was produced with 0.1 TCID₅₀ per cell, and therefore this multiplicity was used in subsequent experiments. Significant amounts of interferon were consistently present at 24 and 48 hr (Table 1) after inoculation with parainfluenza-3 virus. Under the experimental conditions employed, a maximum of 99% reduction in virus yield from interferon-treated cells was observed. A corresponding reduction in the cell death of challenged macrophages also occurred (Table 2).

In occasional experiments, the extracts obtained from noninfected control macrophage cultures conferred interferon-like activity on recipient macrophages. These effects were usually slight and showed animal to animal variation. It would not be surprising to find low levels of interferon-like activity in cells obtained from the respiratory tract of rabbits encountering microorganisms in their natural environment. Accordingly, rabbits having a naturally occurring respiratory infection caused by *Bordetella bronchiseptica*

TABLE 1. *Interferon production in rabbit alveolar macrophages**

Time after inoculation with parainfluenza-3 virus	Plaque reduction of rabbitpox virus
hr	%
12	0
18	29
24	97
48	97
72	70
Control	0

* The data presented in this table represent one typical experiment from a series of nine experiments. Alveolar macrophages were inoculated with 0.1 TCID₅₀ of parainfluenza-3 virus per cell. At the designated intervals, samples were removed and assayed for interferon. Alveolar macrophages were incubated in the interferon preparations for 18 hr and challenged with 0.1 PFU of rabbitpox virus per cell. After 24 hr of incubation, samples were assayed for infectious rabbitpox virus in HEp-2 cells. The per cent plaque reduction was calculated by dividing the difference between mean control and mean test counts by control counts times 100.

were studied with respect to interferon-like activity associated with their alveolar macrophages. Several experiments of this type failed to reveal the presence of interferon-like activity.

In view of the report that homologous nucleic acid can, under certain conditions, induce interferon activity in cells (20), it appeared desirable to determine whether or not dead alveolar macrophages or their products could induce interferon-like activity. This possibility warranted special attention, since it was routinely observed that living macrophages removed small numbers of dead cells from the cultures. Experiments were conducted in which suspensions of killed macrophages were added to an equal number of living cells. Incubation was carried out for 24 to 48 hr. No evidence was obtained suggesting that, under the conditions of these experiments, interferon-like activity was induced in living macrophages by dead alveolar macrophages or their breakdown products.

Properties of the macrophage interferon. Experiments were performed to determine whether the inhibitor possessed the properties of the prototypic interferon described by Isaacs and Lindemann (8). Representative interferon preparations were subjected to the following tests.

Acid stability and dialyzability. Crude interferon prepared in macrophages was dialyzed at 4 C against 100 to 200 volumes of saline, adjusted to pH 4 with 1 N HCl, for 18 to 24 hr, and was

TABLE 2. *Effect of interferon on virus yield and viability of alveolar macrophages infected with rabbitpox virus**

Treatment of macrophages	Per cent cell viability		No. of plaques		Per cent plaque reduction	
	24 hr†	48 hr	24 hr	48 hr	24 hr	48 hr
Extract of parainfluenza-3 infected macrophages (interferon).....	95	82	9	9	97	98
Extract of normal macrophages..	80	50	250	373	5	12
Virus-control.....	76	43	262	423	0	0
Uninfected cell control.....	98‡	96‡	0	0	0	0

* For experimental protocol, see footnote to Table 1.

† Hours after challenge with 0.1 PFU of rabbitpox virus per cell.

‡ Not challenged with rabbitpox virus.

back-dialyzed overnight against Hanks' BSS (pH 7.6). The activity of the extracts was not reduced, indicating that the interferon-like inhibitor was nondialyzable and stable at pH 4.

Extracellular inactivation of virus. When 100 PFU of rabbitpox virus was incubated with undiluted interferon or control fluid for 1 hr at 37 C, the amount of infectious virus recovered from the two suspensions was the same. Thus, the interferon preparation did not directly inactivate the virus.

Species specificity. Primary green grivet monkey kidney cells were employed to determine whether the inhibitor exhibited species specificity. The cells were pretreated for 6 hr with a 1:2 dilution of the inhibitor and challenged with 100 PFU of rabbitpox virus. After 48 hr of incubation, approximately 75% of both the interferon-treated cells and virus control cells underwent cytopathogenesis. Extracts of the monkey kidney cells were assayed in HEp-2 cells. There was no reduction in virus titer in the interferon-treated cells, indicating that the rabbit macrophage interferon did not protect cells of another species against rabbitpox virus.

These data (Table 3) indicate that the interferon induced in alveolar macrophages by parainfluenza-3 virus exhibits the major properties of previously described interferons.

DISCUSSION

The review by Mims (15) of the pathogenesis of viral diseases summarized numerous investigations on the interaction of viruses and the RES. In the case of nonpathogenic viruses, cells of the RES probably play an important role in clearance and disposal of viral particles. On the other hand, pathogenic viruses appear to be resistant to this process and possibly succeed in using cells of the RES for replication or as vehicles for transport to other anatomical sites, or both.

TABLE 3. *Properties of macrophage-produced interferon*

Criterion	Macrophage interferon	Classical interferon
Dialyzable.....	—	—
Stability at pH 4.....	+	+
Sedimented at 100,000 × g.....	—	—
Species-specific.....	+	+
Active against heterologous virus.....	+	+

The work of Kono and Ho (11) lends support to the concept that cells of the RES may be involved in interferon immunity. They observed that tissues containing large numbers of phagocytic cells (i.e., spleen) formed interferon more rapidly and in higher titer than did companion tissues with lower populations of phagocytes (i.e., kidney).

Glasgow and Habel (3) found that interferon was produced by peritoneal macrophages following both in vitro and in vivo infection with vaccinia virus. They established that passively transferred leukocyte-produced interferon contributed to the attenuation of the course of a vesicular stomatitis virus infection in mice. Glasgow (2), employing an in vitro system, observed that normal mouse peritoneal macrophages inhibited the progression of vaccinia virus infections in mouse embryo fibroblast cultures. He suggested that the protective effect could be due to interferon production by the macrophages as a result of their infection with vaccinia virus.

Isaacs and Hitchcock (7) reported that interferon was produced in the lungs of nonimmune mice infected with a sublethal dose of influenza virus, and postulated that interferon may have promoted recovery from the infection.

The present study establishes that rabbit alveo-

lar macrophages produce an interferon after inoculation with parainfluenza-3 virus in vitro. This interferon can passively protect other alveolar macrophages from infection with virulent rabbitpox virus. In this regard, Gresser and Enders (5) demonstrated that a small population of virus-resistant interferon-producing cells interspersed in a large population of susceptible cells altered the course of a sindbis virus infection. Accordingly, interferon produced by a few alveolar macrophages as a result of infection with viruses of low virulence could enhance the resistance of the host by passively immunizing neighboring cells against superinfection with more virulent viruses. The production of interferon could also attenuate or promote recovery from an existing viral respiratory infection. The unique anatomical location of alveolar macrophages, coupled with their almost certain important role in the phagocytosis of microorganisms, makes them prime candidates for interferon production during the initial stages of a viral respiratory infection.

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LITERATURE CITED

1. BARON, S., C. E. BUCKLER, R. M. FRIEDMAN, AND R. V. McCLOSKEY. 1966. Role of interferon during viremia. II. Protective action of circulating interferon. *J. Immunol.* **96**:17-24.
2. GLASGOW, L. A. 1965. Leukocytes and interferon in the host response to viral infections. I. Mouse leukocytes and leukocyte-produced interferon in vaccinia virus infection in vitro. *J. Exptl. Med.* **121**:1001-1018.
3. GLASGOW, L. A., AND K. HABEL. 1963. Interferon production by mouse leukocytes in vitro and in vivo. *J. Exptl. Med.* **117**:149-160.
4. GRESSER, I. 1961. Production of interferon by suspensions of human leukocytes. *Proc. Soc. Exptl. Biol. Med.* **108**:799-803.
5. GRESSER, I., AND J. F. ENDERS. 1962. Alteration of cellular resistance to sindbis virus in mixed cultures of human cells attributable to interferon. *Virology* **16**:428-435.
6. HITCHCOCK, G., AND A. ISAACS. 1960. Protection of mice against the lethal action of an encephalitis virus. *Brit. Med. J.* **2**:1268-1270.
7. ISAACS, A., AND G. HITCHCOCK. 1960. Role of interferon in recovery from virus infections. *Lancet* **2**:69-71.
8. ISAACS, A., AND J. LINDENMANN. 1957. Virus interference. I. The interferon. II. Some properties of interferon. *Proc. Roy. Soc. (London) Ser. B* **147**:258-267, 268-273.
9. JOKLIK, W. K. 1964. The intracellular uncoating of poxvirus DNA. I. The fate of radioactively labeled rabbitpox virus. *J. Mol. Biol.* **8**:263-276.
10. JONES, B. R., J. E. K. GALBRAITH, AND M. K. AL-HUSSAINI. 1962. Vaccinia keratitis treated with interferon. *Lancet* **1**:875-879.
11. KONO, Y., AND M. HO. 1965. The role of the reticuloendothelial system in interferon formation in the rabbit. *Virology* **25**:162-166.
12. LIU, C. 1955. Studies on influenza infection in ferrets by means of fluorescein-labelled antibody. I. The pathogenesis and diagnosis of the disease. *J. Exptl. Med.* **101**:665-676.
13. MARCUS, P. I., S. J. CIECIURA, AND T. T. PUCK. 1956. Clonal growth in vitro of epithelial cells from normal human tissues. *J. Exptl. Med.* **104**:615-628.
14. McLIMANS, W. F., E. V. DAVIS, F. L. GLOVER, AND G. W. RAKE. 1957. The submerged culture of mammalian cells: the spinner culture. *J. Immunol.* **79**:428-433.
15. MIMS, C. A. 1964. Aspects of the pathogenesis of virus diseases. *Bacteriol. Rev.* **28**:30-71.
16. MYRVIK, Q. N., E. S. LEAKE, AND B. FARISS. 1961. Studies on pulmonary alveolar macrophages from the normal rabbit: a technique to procure them in a high state of purity. *J. Immunol.* **86**:128-132.
17. POLLIKOFF, R., M. LIEBERMAN, AND N. E. LEM. 1965. Role of interferon and partial immunity in influenza virus infection of mouse lung. *Proc. Soc. Exptl. Biol. Med.* **119**:790-793.
18. POSTLETHWAITE, R. 1960. A plaque technique for the titration of vaccinia virus in chick embryo cells and some features of vaccinia infection in this system. *Virology* **10**:466-482.
19. ROBERTS, J. A. 1962. Histopathogenesis of mousepox. I. Respiratory infection. *Brit. J. Exptl. Pathol.* **43**:451-461.
20. ROTEM, Z., R. A. COX, AND A. ISAACS. 1963. Inhibition of virus multiplication by foreign nucleic acids. *Nature* **197**:564-566.
21. VOGEL, J., AND A. SHELOKOV. 1957. Adsorption-hemagglutination test for influenza virus in monkey kidney tissue culture. *Science* **126**:358-359.
22. WAGNER, R. R. 1963. Cellular resistance to viral infection, with particular reference to endogenous interferon. *J. Appl. Bacteriol.* **27**:72-86.
23. WAGNER, R. R. 1964. The interferons: biologic basis for their role in viral infections. *Postgrad. Med.* **35**:512-517.