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Interaction of Influenza Virus with Mouse Macrophages

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Mouse peritoneal and alveolar macrophages differed substantially in their response to influenza in vitro. Immunofluorescent and infectious-center techniques showed that viral proteins were produced in only a small subpopulation (17%) of peritoneal macrophages and that these infected cells were removed from culture by 3 days postinfection. In contrast, alveolar macrophages were highly susceptible to influenza, and viral antigens were produced in all cells. This was accompanied by a cytopathic effect and cell death. However, no infectious virus was released and the infection was considered abortive. With mouse cytomegalovirus, however, both alveolar and peritoneal macrophages were equally restrictive, and viral antigens were produced in only 1 to 5% of either cell population. No significant differences were observed between mouse-virulent and -avirulent strains of influenza in their interaction with macrophages either in vitro or in vivo. In vivo, both strains induced an influx of cells to the alveolar spaces by 3 to 4 days postinfection, and this was reflected by a 5- to 10-fold increase in the number of "macrophages" in harvest fluids at this time. Many of these cells had an altered morphology compared with alveolar macrophages from uninfected mice, and the cell population as a whole was not susceptible to influenza. However, this resistance was lost by 7 days of in vitro culture.

Macrophages are important elements in the body's defenses against infection, having a primary phagocytic role and also being capable of complex interactions with other components of the immune system (16). It has been suggested (11) that, due to their prime position for monitoring the body's fluids and spaces, they may play a crucial role in the outcome of infection. Macrophages, therefore, may be important factors in the pathogenesis of virus disease (13) and have been shown to be restrictive for a number of viruses (1, 12, 22).

In the case of influenza, the macrophages encountered by the virus are the resident alveolar macrophages and the blood monocytes that infiltrate the alveolar spaces during influenzal pneumonia (25). It has long been known that influenza infection of mice predisposes them to secondary bacterial infection (6, 19), and it has been suggested that this is due to the impaired function of these macrophages. Warshauer et al. (23), for instance, have suggested that in influenza-infected mice both uptake and inactivation of bacteria are impaired. Others (9, 21) have shown that, after intranasal infection with Sendai virus, phagocytosis of *Staphylococcus aureus* is normal but intracellular killing is impaired. This is supported by recent work (10) demonstrating a defect in phagosome-lysosome fusion in Sendai virus infection. However, Nugent and Pesanti (17), using an in vitro system, have failed to show any alterations in phagocytosis, phagolysosome formation, or bacterial killing in alveolar macrophages after infection with a mouse-adapted strain of PR8 influenza.

In this paper we examine the interaction of influenza virus with mouse peritoneal and alveolar macrophages in vitro. Also, we look for a possible role for these cells in the pathogenesis of influenza infection by means of in vivo studies, comparing the behavior of virus strains that are virulent or avirulent for mice.

MATERIALS AND METHODS

Virus. Four strains of influenza virus were used in this study: A/PR/8/34(H0H1) and NWS(H0H1), kind gifts from J. Oxford, National Institute for Biological Standards Control, Hampstead, England; and two strains of Kunz influenza A(H1N1), designated M0 and M25, kind gifts from R. Heath, Department of Virology, St. Bartholomew's Hospital, London, England. Both Kunz strains were derived from the same original isolate, but strain M0 has since been propagated only in eggs, whereas strain M25 has been mouse adapted by 25 serial passages in the mouse lung. The two strains now differ widely in their virulence for adult CD1 mice (see Fig. 1).

Stocks of all strains were propagated in the allantoic cavity of 10-day-old embryonated hen eggs, clarified at 1,000 × *g* for 30 min, and stored in aliquots at −70°C.

In the comparative study of mouse cytomegalovirus in macrophages, a Smith strain of mouse cytomegalovirus, propagated at low multiplicity in mouse embryo fibroblasts, was used. This strain, originally received from the American Type Culture Collection,

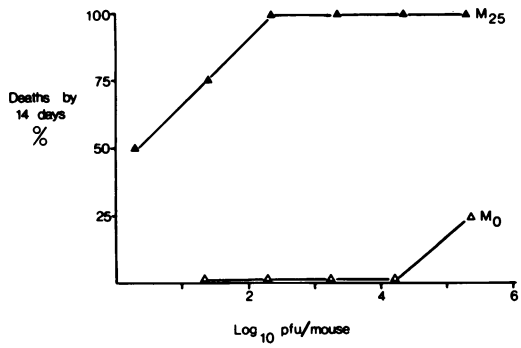

FIG. 1. *Virulence of Kunz influenza for adult CD1 mice. Virus strains: M0, avirulent strain of Kunz influenza virus passed only in eggs; M25, a virulent strain of Kunz influenza virus adapted to mice by 25 serial passages in mouse lungs. Groups of four mice were inoculated intranasally with 0.05-ml amounts of virus, and deaths were scored over a period of 14 days. In addition to deaths in the M25 groups being more numerous, M25-infected mice tended to die much earlier.*

Rockville, Md., was a kind gift from Janine Gould, Department of Microbiology, Guy's Hospital, London, England.

Mice. An outbred, specific-pathogen-free strain of CD1 mice was used for all experiments.

Macrophages. Peritoneal macrophages were obtained by lavage of the peritoneal cavity of normal, untreated mice with 5 to 10 ml of Eagle minimum essential medium containing 15% fetal calf serum and 10 U of heparin per ml. Cells were then placed in ring cultures (2), allowed to settle for 4 to 5 h, and then washed three times with phosphate-buffered saline to remove nonadherent cells. The adherent cells remaining were subsequently kept at 37°C in Eagle minimum essential medium containing 15% fetal calf serum in a 5% CO_2 humidified incubator. These cells were considered to be macrophages, and >90% showed phagocytosis of latex beads and bacteria.

Mouse alveolar macrophages were obtained by lavage of the lungs via the trachea with 2 to 3 ml of the same medium. They were then dispensed to ring cultures, washed, and maintained in the same way as peritoneal cells. More than 75% of these cells phagocytosed latex beads and bacteria.

Unless otherwise stated, macrophage cultures were kept at 37°C for 3 days before use in experiments.

Infection of mice. Mice were inoculated with influenza intranasally under light anesthesia, using 0.05 ml of virus dilution per mouse. Any mice blowing the inoculum back were discarded.

Infection of macrophages. Cultures of macrophages in rings were infected by addition of appropriate dilutions of the virus in medium. After a 1-h adsorption period the inoculum was removed, and the cells were maintained at 37°C in Eagle minimum essential medium containing 15% fetal calf serum.

Virus assay. Influenza virus was titrated by plaque assay, using a modification of the method of Gausch and Smith (4). Briefly, this involved titrating the virus on confluent cultures of canine kidney (MDCK) cells, using a 0.5% agar overlay (Oxoid L.28 purified agar) containing 1.0 U of trypsin (tolylsulfonyl phenylalanyl chloromethylketone; Cambrian Chemicals Ltd., Croydon, England) per ml.

Infectious-center assay. For the estimation of infectious centers, 10^6 plaque-forming units (PFU) of influenza per ring were added to duplicate ring cultures of macrophages, and after adsorption for 1 h, the inoculum was removed and the cells were washed three times with phosphate-buffered saline to remove any residual virus. Medium was replaced, and after incubation at 37°C for appropriate times, the cells were washed three more times in phosphate-buffered saline and gently scraped into a small volume of Eagle minimum essential medium containing 5% fetal calf serum before being seeded onto confluent monolayers of canine kidneys (MDCK) cells. After 30 min of adsorption, the cultures were overlaid with agar containing trypsin as above, and plaques were counted after 2 days of incubation at 37°C.

Supernatant fluids from the final cell washings were assayed for free infectious virus, and these titers were taken into account when determining numbers of infectious centers.

Fluorescent-antibody staining. Immunofluorescent staining for viral antigens was carried out by the indirect staining method, and in most experiments hyperimmune mouse anti-Kunz M25 serum was used. Rabbit antisera to specific components of influenza virus (i.e., hemagglutinin and nucleoprotein) were a kind gift from J. Oxford. Rabbit anti-NS1 antiserum was a kind gift from N. Dimmock, Department of Biological Sciences, University of Warwick, Coventry, England.

Appropriate goat anti-rabbit and goat anti-mouse fluorescein isothiocyanate conjugates were supplied by Nordic Immunological Laboratories, Maidenhead, Berkshire, England.

Fixed cell monolayers were prepared for staining by rapidly drying them in air and treating them in acetone for 10 min at room temperature. Living cells were stained for surface antigen after rinsing with phosphate-buffered saline and maintaining them at 4°C for 10 min. All subsequent staining and washing procedures were conducted at 4°C. Stained monolayers were observed with a Leitz Orthoplan microscope equipped for epi-illumination with an HBO 200 lamp and water immersion objectives.

RESULTS

In vitro studies. (i) Peritoneal macrophages: immunofluorescent studies. In initial studies the NWS strain of influenza was added to peritoneal macrophages at 10^6 PFU/ring (multiplicity of infection, 5 to 10). Fluorescent-antibody staining for viral antigens showed a maximum of 17% of these cells to be positive at 8 h and a subsequent decline to <0.1% positive at 72 h (Table 1). This time course of events was also seen when cells were stained with antisera specific for hemagglutinin and nucleoprotein and

TABLE 1. *Immunofluorescent staining of peritoneal macrophages infected with NWS virus*[a]

Time of staining (h)	% Fluorescent-antibody positive[b]
4	8
8	17
12	12
16	4
19	1.5
22	0.8
48	0.5
72	<0.1

[a] Cells were infected with 10^6 PFU/ring and stained with antiserum raised against the whole virus at the times stated. Staining for hemagglutinin and nucleoprotein alone gave similar results.

[b] Mean value from counts on two rings.

for all virus strains tested (NWS, PR8, Kunz M0, Kunz M25). In the case of the Kunz viruses these results were supported by hemadsorption (unpublished data) and infectious-center data (Table 2), both of which showed a similar rise and fall in the number of infected cells. No virus release into the medium was detected in any of the infected macrophage cultures.

These results are compatible with those of Shayegani et al. (20) and suggest that a single cycle of replication occurs in a limited subpopulation of peritoneal macrophages. Further experiments were performed to establish whether this was a true subpopulation and to determine the fate of viral antigens in the decline period (8 to 72 h).

Staining observations. Careful examination of stained cultures showed that brightly fluorescing infected cells were often surrounded by macrophages containing antigen only in vesicles (Fig. 2), suggesting that the antigen-containing material from the degenerating primarily infected cell is phagocytosed by its neighbors. Trypan blue staining revealed that nonviable cells were usually surrounded by a tight ring of viable macrophages and that the number of nonviable cells varied with time in the same way as the number of fluorescent cells, with a peak of 10% nonviable at 8 h and a decline to only 2% at 24 h.

Reinfection experiments. Cultures of peritoneal macrophages were exposed to 10^6 PFU of strain NWS virus per ring and then, 3 days later, reinfected with the same dose of virus for 8 h. Only 1% of the cells treated in this way contained viral antigen compared with 17% in the control, which was infected for only the final 8 h. This suggests a susceptible subpopulation which is exhausted by the first exposure to the virus.

Dose-response experiments. Dose-response experiments were performed with both the virulent and the avirulent strain of Kunz influenza virus in peritoneal macrophages. These studies revealed no significant differences between strains, but did show that with increasing input multiplicities a plateau was reached at which no more cells could be infected despite the use of high virus titers (Fig. 3). This provides further evidence of a susceptible subpopulation.

(ii) Alveolar macrophages: immunofluorescent studies. In contrast to peritoneal cells, fluorescent-antibody studies showed that resident alveolar macrophages were uniformly susceptible to influenza. Infection with 10^6 PFU of strain PR8, strain NWS, or either Kunz strain per ring resulted in viral antigens being produced in all cells by 16 h. This was accompanied by a gross cytopathic effect with severe cell rounding. Trypan blue staining showed that at 16 h all cells were nonviable. No virus release was detected over a period of 3 days, and so the infection was considered abortive.

The pattern of fluorescent staining was similar with all virus strains tested (PR8, NWS, and Kunz), with viral antigens appearing first in the nucleus at 4 h and later in the cytoplasm and with a densely staining intranuclear inclusion developing by 16 h (Fig. 4). Further immunofluorescent studies with antisera to specific viral components showed that nucleoprotein, hemagglutinin, and NS1 proteins are synthesized in infected cells. Staining of living cells at 4 to 6 h showed that nucleoprotein and hemagglutinin also appear on the surface of infected macrophages. The appearance of hemagglutinin on the surface is supported by hemadsorption studies, which showed that 70 to 95% of infected macrophages will hemadsorb human O erythrocytes by 8 h postinfection.

Mouse cytomegalovirus. The above studies show a clear difference between alveolar and peritoneal macrophages in their response to in-

TABLE 2. *Comparison of the number of infectious centers in cultures of peritoneal macrophages at various times after infection with Kunz influenza virus*

Time postinfection (h)	No. of infectious centers per ring[a]	
	M0	M25
0	10	20
8	594	582
24	90	165
72	0	15
96	0	0

[a] Values are means of four cultures and have been corrected to take into account free virus remaining in the fluids.

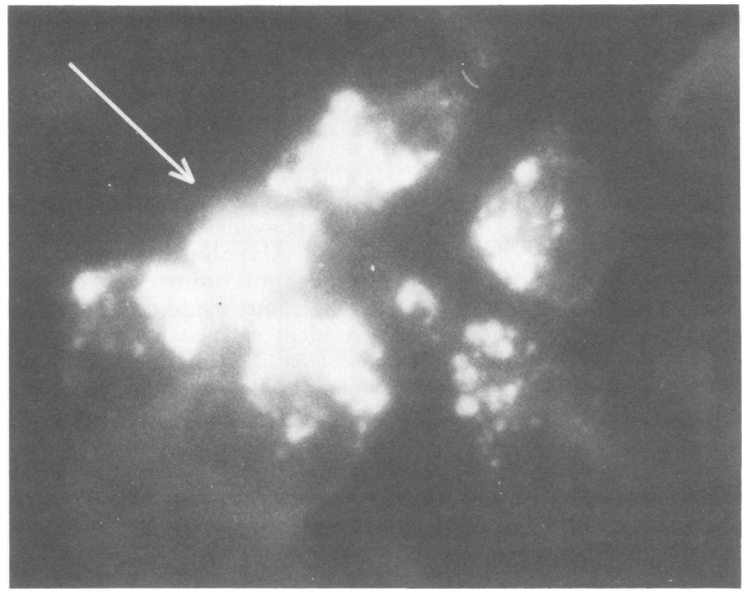

FIG. 2. *Influenza-infected peritoneal macrophages stained for viral antigens at 16 h postinfection. Degenerate infected macrophage (arrow) surrounded by uninfected cells phagocytosing antigen. ×1,000.*

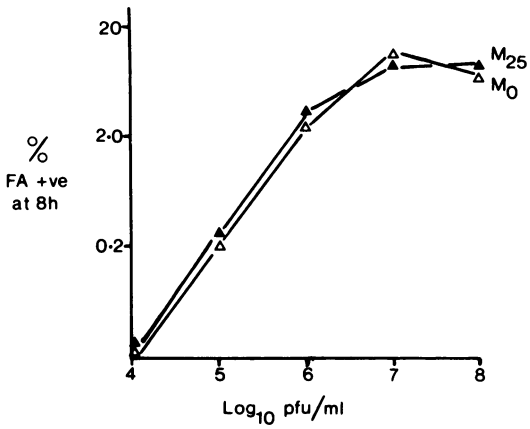

FIG. 3. *Dose response of Kunz influenza virus strains in peritoneal macrophages. FA +ve, Fluorescent-antibody positive.*

fluenza virus. In comparative studies with mouse cytomegalovirus such differences between cell types were not apparent. When ring cultures of peritoneal and alveolar macrophages were infected with 10^4 to 10^5 PFU of Smith strain mouse cytomegalovirus per ring, only 1 to 5% of the cells in either macrophage population became infected as indicated by immunofluorescence. Viral antigens first appeared in these cells at 24 h and were maintained over a period of 4 days.

In vivo studies. In vivo studies were carried out with the virulent (M25) and avirulent (M0) strains of Kunz influenza virus to examine the involvement of macrophages in the pathogenesis of influenza infection. With an initial inoculum of 10^4 PFU/mouse, two very different infections were produced. In the case of the avirulent strain (M0) mice suffered little or no macroscopic lung consolidation and usually recovered, whereas the virulent strain (M25) produced a severe, often fatal disease manifested by gross weight loss, ruffled fur, and extensive lung consolidation.

Immunofluorescent studies. Macrophages lavaged from infected mice were separated from nonadherent cells 2 h after harvest and immediately fixed for staining to avoid artifacts arising from infection due to high extracellular virus titers (10^6 to 10^8 PFU/ml) present in the lavage fluids. These studies showed that, during the first 16 to 24 h of infection, only 1 to 12% of the macrophages contained viral antigens. This figure is very low compared with the 100% infection seen in vitro. However, there was no increase in this figure when larger inocula (10^5 or 10^6 PFU/mouse) were given, and therefore we believe that the low number of infected cells reflects that influenza infection of the lung is focal and that many macrophages are lavaged from uninfected segments. The cells harvested at these times were all susceptible when challenged with 10^6 PFU of influenza per ring in vitro. No significant differences between the two strains were noted in these studies.

At later times, from 3 to 7 days postinfection, there was a 5- to 10-fold increase in the number

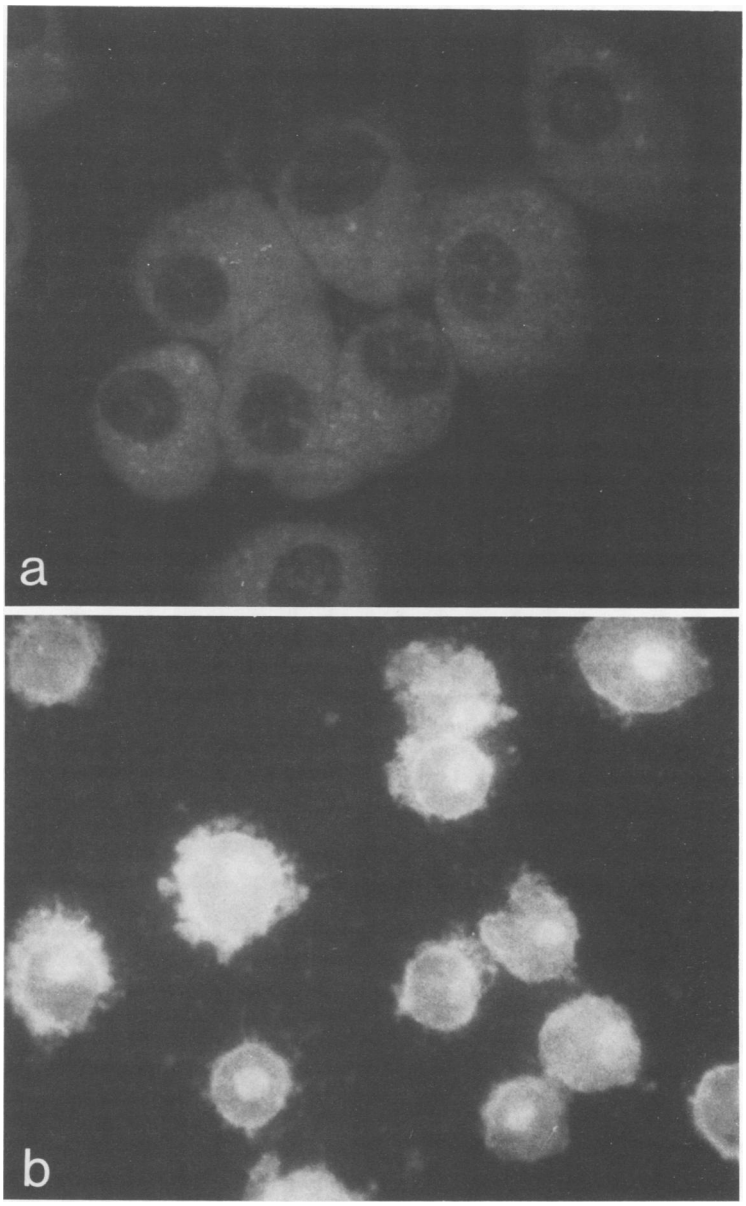

FIG. 4. *Immunofluorescent staining of alveolar macrophages infected with Kunz influenza virus. (a) uninfected macrophages; (b) 16 h postinfection (note shrunken cytoplasm and densely staining intranuclear inclusion).* ×*1,000.*

of macrophages in lavage fluids from both M0- and M25-infected mice. Cultures of adherent cells obtained at these times differed from those from uninfected mice in that there was a much greater heterogeneity of cell size, with cells often arranged in loosely associated "islands." Also, some of these cells spread out more quickly on glass. Immunofluorescent staining at 2 h after harvest showed that only 1% of these cells exhibited the characteristic pattern of staining seen in vitro. However, up to 80% contained viral antigens in a pattern suggesting phagocytosis of infected debris. Staining of these cells for mouse immunoglobulin was negative. After maintenance in vitro for 3 days, cells from both M0- and M25-infected mice were spread out and viable, despite the presence of $>10^7$ PFU of virus per ml in the lavage fluids, and the phagocytosed

debris was no longer apparent. This suggests that the macrophage population present in the lung at this time is resistant to influenza infection.

Superinfection experiments. To determine whether macrophages from infected mice were resistant to influenza infection, mice infected 4 days previously with 10^4 PFU of M0 or M25 virus per animal were lavaged, and the adherent cells were exposed to 10^6 PFU of either Kunz strain per ring in vitro. This amount of virus produces infection in all cells from uninfected mice. If challenged at 5 h after harvest, nearly all of the adherent cells from infected mice were resistant, but if challenged after 7 days of in vitro culture, nearly all were susceptible (see Table 3).

DISCUSSION

This study has outlined major differences between peritoneal and alveolar macrophages in their response to influenza virus. Uninduced peritoneal macrophages of mice are largely resistant to influenza infection, exhibiting only limited, transient replication in a subpopulation (17%) of cells. This indicates that peritoneal macrophages do not represent a totally homogeneous cell population. The biological relevance of the susceptible cell fraction is as yet unclear. In contrast, all resident alveolar macrophages appear to be highly susceptible to influenza, with viral proteins being produced in all cells by 8 h postinfection. By 16 h there is a gross cytopathic effect, severe cell rounding and loss of viability by trypan blue staining. The development of cytopathic effect was seen with Kunz, NWS, and PR8 strains of virus, and it is assumed that phagocytic and other functions are impaired under these conditions. These results are contradictory to those of Nugent and Pesanti (17), but the differences may be attributable to the different strain of mice (CF1) used or the fact that their macrophages were harvested by collagenase digestion of whole lungs. There have been no previous reports of differences in virus susceptibility of macrophages from different body sites, but differences are perhaps to be expected, as macrophages from different sites are known to vary considerably in biochemistry and metabolism (3, 14). Interestingly, however, the result with mouse cytomegalovirus indicates that both peritoneal and alveolar macrophages are resistant to this virus.

TABLE 3. *Resistance of lung macrophages from influenza-infected mice to superinfection in vitro*

Time after harvest of in vitro challenge	Source of macrophages	Infection in vitro (PFU, strain)	% Fluorescent-antibody positive (at 16 h postinfection)
5 h	Uninfected mice	10^6, M0	100
	Infected mice	Nil	<0.1
	Infected mice	10^6, M0	3
	Infected mice	10^6, M25	11
7 days	Uninfected mice	10^6, M0	100
	Infected mice	Nil	<0.1
	Infected mice	10^6, M0	90
	Infected mice	10^6, M25	95

The lung macrophages obtained 4 days after intranasal infection with the Kunz strains are resistant to influenza and so are markedly different from the resident alveolar cells of uninfected mice. The basis of this resistance is not yet clear, but a number of possibilities exist. First, the population of lung macrophages in infected mice may be "activated" and, therefore, more resistant to infection, a phenomenon seen in other systems (7, 15). Also, macrophages harvested at these times may have been exposed to other factors such as antibody or interferon, and their virus susceptibility may have altered as a result. Wells et al. (24), for instance, have shown that addition of antibody to influenza-infected macrophages can reduce the number of infectious centers. Haller and co-workers (5) have shown that macrophages from genetically susceptible mice can be rendered resistant to influenza by treatment with interferon. Finally, it is possible that most of the cells seen at 4 days after infection represent the newly arrived monocytes described by Wyde et al. (25) and that these cells behave differently from the resident alveolar cells in their respose to influenza.

In these studies we have compared the behavior in macrophages of a virulent and an avirulent variant of Kunz strain influenza. Other workers, using these virus strains, have shown (8) that cyclophosphamide treatment of mice will greatly increase mortality with the avirulent strain, implying that cellular responses may be important in controlling this infection. The two strains of virus were also seen to give similar serum-antibody and interferon responses after intranasal infection of mice (18). It was concluded that the difference in pathogenicity could not be attributed to nonspecific inhibitors or inadequate antibody responses to the virulent strain. It was also noted that macrophages appeared to support viral replication, but this was not firmly established. In the experiments reported above we have shown that resident alveolar macrophages can support replication of these viruses in vitro and that infected macrophages are pres-

ent in lung washings from Kunz-infected mice. However, no differences were detected between the two strains either in vitro or in vivo. Thus, in this case virulence does not appear to be macrophage mediated. However, differences between the two strains in their interaction with macrophages may still exist, as the interaction of these strains with the resistant macrophages present at 4 days postinfection has yet to be studied in detail.

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