INFECTION AND IMMUNITY, Nov. 1979, p. 651-657 Vol. 26, No. 2
0019-9567/79/11-0651/07$02.00/0

Effect of Influenza Infection on the Phagocytic and Bactericidal Activities of Pulmonary Macrophages

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Received for publication 20 August 1979

The effect of mouse-adapted influenza A/PR/8/34 virus on pulmonary macrophage function was evaluated by using an in vitro system which allowed direct virus interaction with macrophages and then separate analysis of the steps required for bacterial clearance by macrophages. Infection of macrophages with this virus resulted in the appearance of a hemagglutinating activity on the macrophage surface; expression of this activity was inhibited by amantadine, 2-deoxyglucose, and cycloheximide and by pretreatment of the virus inoculum with ultraviolet light and specific antiserum. Since there was no release of extracellular virus, this growth cycle appeared to be incomplete (abortive). After influenza infection, net ingestion of viable *Staphylococcus aureus* by macrophage monolayers was unaltered and there was no change in the fraction of the monolayer which ingested cocci over a wide range of bacterial inputs. Influenza-infected macrophages also inactivated intracellular *S. aureus* at a rate indistinguishable from controls. Therefore, these in vitro studies do not support the hypothesis that the defect in pulmonary antibacterial mechanisms associated with influenza infections results from a direct effect of virus infection on either the phagocytic or bactericidal activity of resident pulmonary macrophages.

Influenza infections transiently suppress pulmonary antibacterial defense mechanisms and cause an increased susceptibility to bacterial pathogens. For example, staphylococcal pneumonia is rare in healthy adults except during periods of epidemic influenza. The mechanism underlying this virus-induced alteration in host defenses has been extensively investigated in a murine model for experimental pneumonia developed by Green and Kass (8). In this model, resolving Sendai virus infections suppressed pulmonary antibacterial activity against *Staphylococcus aureus* in both consolidated and nonconsolidated regions of the lung but did not impair physical removal of the inhaled bacteria from the lung (10, 11). These results suggest that the major alteration in host defense involved macrophage function at the alveolar level and not mucociliary transport. However, subsequent studies have not conclusively identified the predominant defect in macrophage function. Jakab and Green, using semiquantitative histological techniques, found that phagocytosis was normal but that intracellular killing was impaired during Sendai infection (12). However, similar studies by other investigators with influenza virus indicated that both phagocytic and bactericidal activity were suppressed (23). In order to more directly analyze these functional defects, we evaluated the phagocytic and bactericidal function of pulmonary macrophages infected in vitro with a mouse-adapted influenza virus.

MATERIALS AND METHODS

Mice. Outbred female CF_1 mice (Carsworth Farm) weighing 20 to 25 g were used throughout. These animals were housed in groups of 10 and allowed free access to mouse chow and water.

Macrophage preparation. Mice were sacrificed by air embolus through tail vein injection and exsanguinated by transection of intrathoracic arteries and veins. Lungs were then aseptically removed, minced into fine pieces with scissors, and digested with a 0.1% collagenase solution (type II [Sigma Chemical Co.] in heparinized medium 199 [M199]) at 37°C for approximately 75 min with constant stirring. This suspension was then filtered through fine-mesh wire and distributed into Leighton tubes. After incubation for 1 h, nonadherent cells and debris were removed with repeated rinses with warm M199. Adherent cells were then incubated in tissue culture medium containing M199, 20% heat-inactivated (56°C for 60 min) newborn calf serum, gentamicin (10 μg/ml), and penicillin (1,000 U/ml) in a 95% air-5% CO_2 atmosphere at 37°C. After overnight incubation, nonadherent cells were again removed with M199 rinses. This procedure resulted in a population of glass-adherent mononuclear cells (approximately 10^5/Leighton tube), of which more than 98% were viable by trypan blue dye exclusion, more than 90% concentrated neutral red in large cytoplas-

mic vacuoles, and more than 95% ingested *S. aureus* when exposed to a heavy inoculum (>10^8 colony-forming units/ml). Preparations with greater than 5% fibroblasts by morphological criteria were discarded.

Influenza virus. Influenza virus A/PR/8/34 (H_0N_1); (obtained from R. M. Massanari, Department of Medicine, University of Iowa Hospitals) was adapted to CF_1 mice by serial respiratory tract infections (25). After the 10th mouse passage, stocks were grown in 11-day-old embryonated eggs. Allantoic fluid was clarified by centrifugation (2,000 rpm for 15 min in a PR6 International centrifuge), stabilized with 1/10 volume of heat-inactivated newborn calf serum, and stored at −70°C in small ampoules. These stocks contained >$10^{5.3}$ 50% mouse lethal dose units per ml. Hemagglutination titers and hemagglutination inhibition titers were determined by using O^+ human erythrocytes (RBC) and standard microtiter technique (19). Fifty percent egg infectious dose titers (EID_{50}) were determined by injecting 0.2 ml of virus dilution into 11-day-old embryonated eggs, incubating the eggs for 48 h at 35°C, and testing allantoic fluid (diluted 1:10) for hemagglutinating activity. Calculations were made with the Reed-Muench approximation (18). The allantois-on-shell assay described by Fazekas de St. Groth et al. was used to detect infectious virions in monolayer supernatants (5, 6). This procedure was modified to ultilize 96-well tissue culture plates and M199 with 0.5% albumin bovine serum. For some experiments, virus stocks were irradiated with a General Electric germicidal light (15 W) at a distance of 12 cm for 8 min in phosphate-buffered saline, pH 7.4 (PBS), with intermittent stirring. Metabolic inhibitors and amantadine were purchased from Sigma Chemical Co., St. Louis, Mo. Antibody to influenza virus was raised in rabbits by using a standard immunization protocol (15). The gamma globulin fraction of this preparation had a hemagglutinin inhibition titer of 100 against 4 hemagglutinin units of virus.

Macrophage infection with virus. Monolayers were exposed to approximately 5×10^7 EID_{50} of influenza virus per ml in M199 (plus 1% albumin) for 1 to 3 h, washed three times with warm M199, and then incubated in our routine tissue culture medium.

RBC rosette technique. The hemadsorption technique described by Vogel and Shelokov (22) was modified to detect the influenza hemagglutinin on the macrophage plasma membrane. At intervals after virus exposure, monolayers were washed twice with warm PBS and then incubated with a suspension of type O^+ human RBC (0.66%, vol/vol) in M199 at room temperature for 30 min. These RBC were collected every 2 to 3 weeks, stored in Alsever solution at 4°C, and washed three times with PBS before use. After incubation with RBC suspension, monolayers were washed three times with PBS and fixed with 1% glutaraldehyde in PBS, and the percent rosette formation (three or more RBC attached) was determined by phase microscopy. Each experiment included uninfected monolayers, and the results reported represent the difference between PR8 and control monolayers.

Macrophage function assays. The usual experimental sequence included in vitro adaptation of macrophages for 24 or 48 h, exposure to influenza virus for 3 h, incubation for 6 or 15+ h after virus exposure, and then evaluation of macrophage phagocytosis, phagolysosome formation, and intracellular bactericidal activity. Phagocytosis of *S. aureus* 502A, subcultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) from stocks maintained on Trypticase soy broth agar plates, was evaluated by quantitative culture techniques and by light microscopic examination of Giemsa-stained cover slips. Phagolysosome formation was assessed by fluorescent microscopic evaluation of acridine orange transfer after ingestion of *Saccharomyces* sp. (commercial bread yeast) (17). Microbicidal activity was evaluated by measuring the viability of monolayer-associated *S. aureus* during prolonged in vitro incubation (2). In brief, after ingestion of *S. aureus* and digestion of extracellular bacteria with lysostaphin, macrophages were incubated in complete medium with 10 U of penicillin per ml. At indicated times, intracellular *S. aureus* were released by hypotonic lysis of the monolayer with distilled water and quantitated with plate counts. The number of viable *S. aureus* was normalized to the number of macrophages per Leighton tube, determined by phase microscopic counts with an eyepiece grid.

Data analysis. Cover slips for phagocytosis and phagolysosome formation assays and Leighton tubes for rosette formation were coded and counted after all data points for a given experiment were collected. At least 200 macrophages were evaluated in each preparation unless indicated otherwise. Data are presented as means ± standard error for results from duplicate slides or tubes from three or more separate experiments. Straight lines were generated by the method of least-mean-squares linear regression analysis. Differences between means and slopes were calculated by Student's *t* test. Differences between macrophage distribution into functional categories were calculated by χ^2 analysis. A two-tailed $P \leq 0.05$ was considered statistically significant.

RESULTS

Interaction between influenza virus and pulmonary macrophages. Since the in vitro interaction between influenza virus and pulmonary phagocytic cells could result in either virus replication or virus ingestion and inactivation, our initial experiments approached these and other less likely alternatives by evaluating influenza-exposed monolayers for evidence of virus replication. This influenza strain did not cause any cytopathic effect in glass-adherent cells over a 7-day period of observation. Furthermore, after virus exposure and removal, there was no detectable release of either infectious virions (allantois-on-shell assay) or hemagglutinating activity (microtiter technique) into the medium during the first 48 h after infection. However, after exposure to influenza virus for 1 to 3 h and subsequent culture in fresh medium, a large fraction of the macrophages hemadsorbed 0^+ human RBC (Fig. 1 and 2). In contrast, washed RBC spontaneously adhered to only 1.4 ± 1.7%

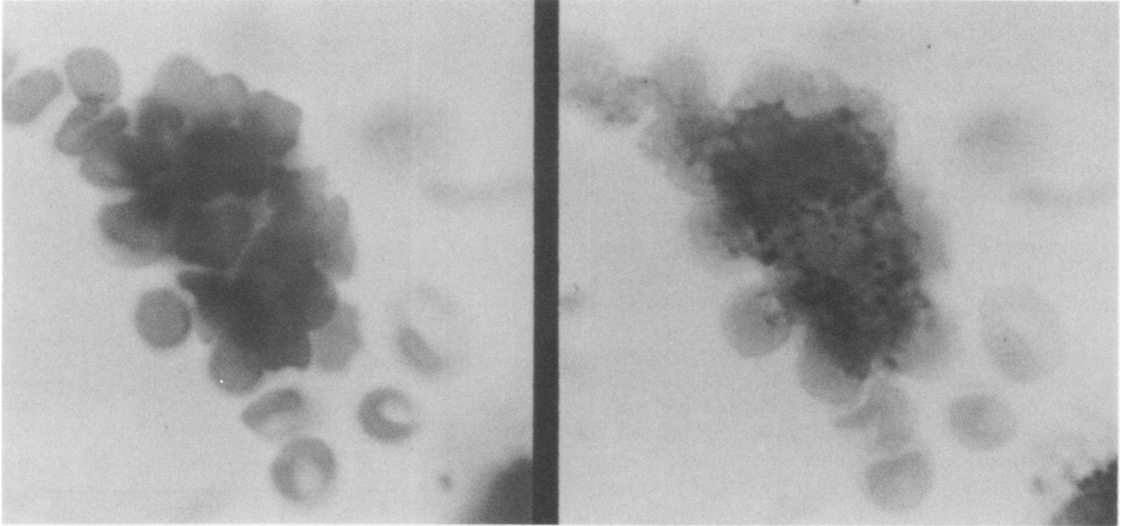

FIG. 1. *Hemadsorption by pulmonary macrophages infected with influenza virus. Macrophages infected with influenza virus were exposed to S. aureus for 60 min, incubated with 0+ RBC for 30 min, washed, fixed with glutaraldehyde, and then stained with Giemsa stain. (A) RBC adherent to macrophage (×400). (B) intracellular S. aureus in the same mononuclear cell (×400 at another plane of focus.)*

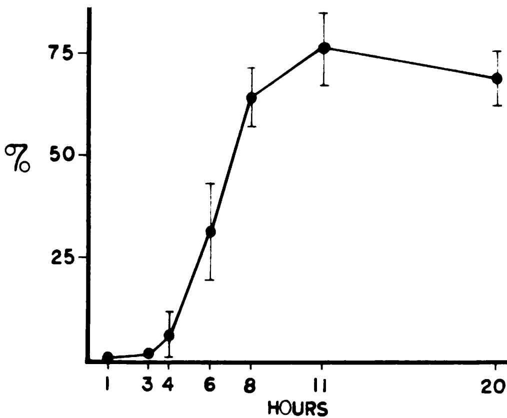

FIG. 2. *Time course for appearance of hemagglutinin on the macrophage surface after exposure to influenza virus. Symbols represent mean ± 1 standard error of the mean.*

(mean ± 1 standard deviation; $n = 50$) of control macrophages. This hemagglutinating activity appeared after a latent period of 4 h, increased to a plateau by 7 to 8 h, and then persisted at a stable level through 24 h (Fig. 2). Subsequently it disappeared with an apparent half-life of 55 h (data not shown). Formation of this hemagglutinin was inhibited when the virus-exposed macrophages were incubated with amantadine (50 μg/ml), 2-deoxyglucose (20 mM), and cycloheximide (2.5 μM) and when the virus inoculum was pretreated with either ultraviolet light or anti-PR8 rabbit serum (Table 1). Since these agents all inhibit influenza replication in other systems (9, 20), these results suggest that the appearance of this hemagglutinating activity required de novo virus-directed glycoprotein synthesis. The presence of virus-directed synthetic activity without release of infectious virions indicates that this was an abortive replication cycle.

Effect of influenza infection on macrophage function. The principal steps involved in microbial clearance by macrophages include ingestion, intracellular killing, and degradation. Although the exact bactericidal mechanism remains unknown, degradation requires exposure of the ingested particle to contents of lysosomes and hence phagolysosome formation. Influenza infections did not change the total accumulation of viable *S. aureus* into macrophages during 1-h ingestion periods (1-h point, Fig. 5). However, since this assay depended on quantitative culture techniques and might not have detected

TABLE 1. *Hemagglutinin formation: inhibitor effects*

Inhibitor	Rosette formation (% of control)[a]
2-Deoxyglucose (20 mM)[b]	0.5 ± 0.5
Amantadine (50 μg/ml)	14.9 ± 6.0
PR8 antiserum (rabbit, 1:10 dilution)	4.6 ± 3.5
Ultraviolet light (15 W, 12 cm, 8 min)	0.3 ± 0.3
Cycloheximide (2.5×10^{-6} M)[b]	45.9 ± 13

[a] All reductions are significant at $P \leq 0.05$.
[b] Added 1.5 h after virus exposure.

functionally defective subpopulations of macrophages in the PR8-infected monolayers, phagocytosis was also evaluated microscopically. The fraction of macrophages which ingested one or more bacteria during exposure to a wide range of bacterial concentrations was measured in PR8-infected and control monolayers, using Giemsa-stained cover slips (Fig. 3). The slopes of the regression lines for the two experimental groups were almost identical (1.135 for infected, $n = 17$, $r = 0.09$; 1.055 for control, $n = 19$, $r = 0.92$), indicating that there was no detectable increase in nonphagocytic cells after virus infection. In three experiments, bacterial ingestion was then more carefully assessed by tabulating the distribution of particle uptake per macrophage. At all three bacterial concentrations tested in these particular experments, the control monolayers had a slightly larger fraction of macrophages which did not ingest any bacteria and a smaller fraction which ingested >10 cocci when compared with influenza-infected monolayers ($P < 0.05$) (Fig. 4). Rosette assays after ingestion of bacteria clearly demonstrated that cells with surface hemagglutinin could ingest a large number of *S. aureus* (Fig. 1).

In the preceding experiments, macrophages had been infected with virus at least 15 h before the phagocytosis assays. However, the most rapid change in hemagglutinin appearance occurred 4 to 7 h after virus exposure (Fig. 1). Therefore, phagocytosis was reevaluated 6 h after virus exposure, using a bacterial input calculated to allow 50% of the macrophages to ingest particles. We found that 37 ± 7% of control and 52 ± 6% of influenza-infected macrophages ingested *S. aureus* during a 1-h exposure. This apparent increased phagocytic activity of PR8-infected macrophages did not reach statistical significance ($P > 0.1$). Thus, influenza infection established in vitro did not affect the initial step (ingestion) in bacterial clearance by macrophages at either time point studied after virus exposure.

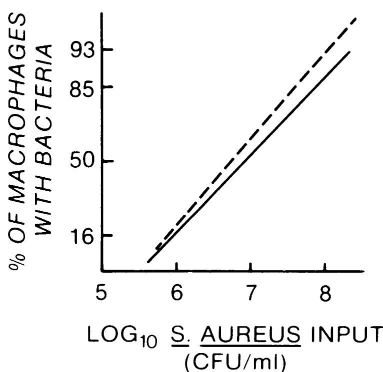

FIG. 3. *Phagocytosis by PR8-infected macrophages and control macrophages. The percentage of macrophages which ingested S. aureus was determined by microscopic examination of Giemsa-stained cover slips and plotted on a probit scale against the log of the bacterial concentration. Symbols:* (- - -) *influenza-infected monolayers;* (——) *control monolayers. CFU, Colony-forming units.*

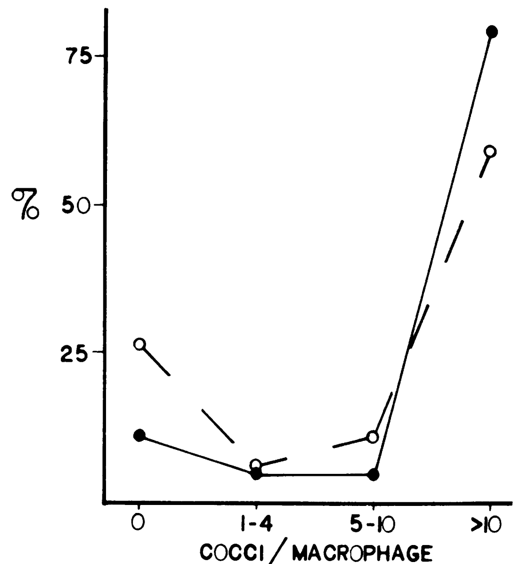

FIG. 4. *Distribution of S. aureus uptake in monolayers of pulmonary macrophages. Monolayers were exposed to S. aureus (7×10^7 colony-forming units/ml) for 1 h and then processed as described in the text. Symbols:* (○) *control monolayers;* (●) *influenza-infected monolayers.*

When compared with reported neutrophil activity (4), pulmonary macrophages display relatively weak bactericidal activity against *S. aureus* (14). Therefore, evaluation of intracellular killing required prolonged incubation periods using bacteriostatic concentrations of penicillin in the medium (added after ingestion and lysostaphin digestion). Our results with pulmonary macrophages (Fig. 5) were comparable to studies reported by Baughn and Bonventre with peritoneal macrophages (2) and demonstrated that the number of viable cell-associated bacteria fell approximately 50% every 10 h, resulting in over 95% reduction in bacterial viability by 48 h. Reductions to this extent allowed us to realistically evaluate the effect of influenza virus on bactericidal activity, and these experiments (Fig. 5) demonstrated that influenza infections established 15+ h before bacterial challenge did not alter intracellular killing. Since the hemagglutinin disappearance half-time exceeds 48 h, most cells with hemagglutinin continued to be in-

fected throughout the time required for these assays.

Phagolysosome formation was evaluated with a microscopic technique which detects the fusion of acridine orange-labeled lysosomes with phagosomes containing *Saccharomyces* sp. Since influenza infection did not affect the rate of phagolysosome formation (Table 2), this implied that both the rate of ingestion and the rate of intracellular fusion were normal and therefore confirmed the phagocytosis studies described above using another larger particle.

In a limited number of experiments, alveolar macrophages obtained by bronchial lavage (3) were compared with the pulmonary macrophages obtained by mincing and collagenase digestion. There was no difference in the in vitro infection with influenza virus or in the effect of virus infection on phagocytosis (Table 3).

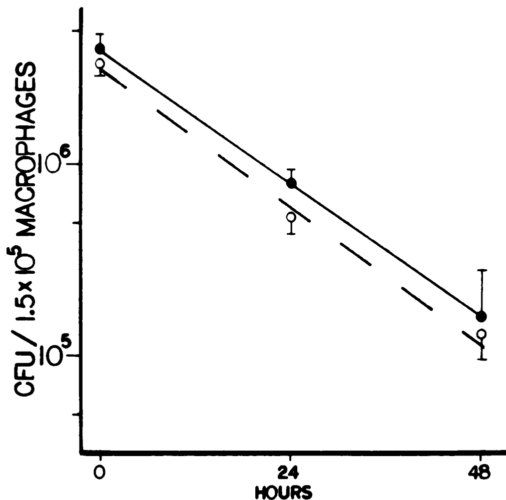

FIG. 5. *Bactericidal activity in influenza virus-infected and control monolayers. The rate of intracellular inactivation of ingested S. aureus by PR8-infected monolayers (●) and control monolayers (O) was determined by following the change in viability of monolayer-associated bacteria with time. Symbols represent mean ± 1 standard error of the mean. CFU, Colony-forming units.*

TABLE 2. *Phagolysosome formation*

Time[a]	Control macrophages	PR8-infected macrophages
25	55 ± 2.5%[b]	62.5 ± 2.3%
60	85.6 ± 3.2%	84.1 ± 0.6%

[a] Minutes after addition of yeast to acridine orange-labeled macrophages.

[b] Percentage of cell-associated yeast with acridine orange stain, determined in three separate experiments.

TABLE 3. *Macrophage preparation*[a]

Method of prepn	Determination		
	% Rosette formation[b]	% Ingesting *S. aureus*[c]	
		Control	PR8 infected
Lavage[d]	57.3	61.5	56.5
Tissue digestion[e]	68.4	56.1	53.8

[a] Results are means of data from two separate experiments.

[b] Determined during plateau phase (see Fig. 2).

[c] $10^{7.93}$ colony-forming units per ml for 1 h.

[d] Using PBS with 1.25 mM ethylenedeaminetetraacetic acid.

[e] See Materials and Methods.

DISCUSSION

Experimental work with murine models of pneumonia has established that resolving viral respiratory tract infections suppress pulmonary antibacterial defense mechanisms. Although this alteration in host defenses appears to reflect impaired macrophage function, both the principal functional defect and the explanation for this reversible process remain unclear (12, 23). We have evaluated the effect of influenza infection on macrophage function, using an in vitro system both for establishing the viral infection and for evaluating macrophage function. This approach allowed separate analysis of phagocytosis and intracellular killing and eliminated other potential virus-associated inflammatory responses which could indirectly alter macrophage function.

Infection of pulmonary macrophages with influenza virus in vitro resulted in the formation of a new hemagglutinin on the macrophage plasma membrane, but did not lead to detectable release of infectious virions into the culture medium. Abortive infections with influenza viruses have been described in other cell lines in detail (1, 7) but have not been reported with macrophages. Shayegani and co-workers demonstrated that influenza A/swine/Iowa/15/31 infected a small percentage of peritoneal macrophages and induced synthesis of a hemagglutinin and the S and V antigens, but they did not test for the release of virions (21). Wells et al. (24) recently reported that in vitro exposure of macrophages to influenza virus does not result in a productive infection. They attributed the block in replication to unsuccessful viral penetration into macrophages and suggested that virus particles were merely carried on the cell surface. In our experiments, however, expression of the hemagglutinin followed a latent period, was in-

hibited by several drugs which inhibit virus-directed synthetic activities, and was abolished by inactivation of virus. These observations are not compatible with a "carrier" role for the macrophages but would be expected in an abortive infection.

When we analyzed hemagglutinin formation as our major indicator of intracellular events during influenza replication (Fig. 2), it seemed appropriate to evaluate phagocytosis during the rapid appearance of hemagglutinin at the cell surface (6 h) and during the stable plateau phase of hemagglutinin formation (15+ h). In these experiments, phagocytosis was evaluated by quantitating the total bacterial uptake into the monolayer, by estimating the fraction of the monolayer involved in ingestion (one or more cocci), and by measuring the relative efficiency of uptake into individual macrophages. Influenza infection did not reduce phagocytic activity in any of these assays. In fact, in some experiments, the trends suggested that virus-infected cells performed more efficiently. Phagolysosome formation measured 15 h after virus exposure was also normal (Table 2).

Our bactericidal assays using *S. aureus* required prolonged incubation to allow measurable reductions in bacterial viability, and this aspect of these assays made it unproductive to compare the survival of bacteria ingested at several time points during the comparatively rapid changes in intracellular events after virus infection. Therefore, we measured the bactericidal activity against *S. aureus* ingested 15+ h after virus exposure and found that the overall rate of killing in these monolayers was not affected by influenza infection (Fig. 5). The bactericidal activity measured in our control monolayers is consistent with results reported for mouse peritoneal macrophages (2). However, LaForce and co-workers evaluated *S. aureus* killing by rat alveolar macrophages and found no intracellular killing (14). The most likely explanation for this discrepancy is the different incubation periods used for the bactericidal assay. We incubated monolayers for 48 h whereas LaForce et al. terminated their assays after 3 h. In our system, a 3-h incubation would have allowed approximately a 20% reduction in the number of viable intracellular bacteria, a reduction that would not have been detectable by plate counting techniques. *S. aureus* was used throughout these assays because of the potential clinical relevance and because lysostaphin allowed the rapid lysis of extracellular organisms necessary for these assays.

In summary, the results from our macrophage function assays indicate that influenza infection does not suppress either phagocytosis or intracellular killing, two important steps in bacterial clearance by macrophages. Mills has reached similar conclusions with pulmonary macrophages infected with Sendai virus (16). However, it is possible that the excessive bacterial ingestion noted in some of the phagocytosis experiments (Fig. 4) may have a detrimental effect on bacterial clearance. Baughn and Bonventre found that the number of bacteria ingested could affect the initial rates of bacterial killing (2). Therefore, the bacterial survival in the subpopulation of macrophages which ingest large numbers of bacteria may be prolonged in our virus-infected monolayers. However, our assay measured overall bactericidal activity in the monolayer and could not detect slightly impaired killing in a subpopulation within the monolayers.

The results described above would not have been predicted from the in vivo animal studies in which both phagocytosis and intracellular killing appeared abnormal (12, 23). This discrepancy may reflect the difficulty in identifying the principal functional defect by using in vivo techniques or in distinguishing among many possible direct and indirect viral effects associated with a viral pneumonitis. Macrophage function may have been altered in the in vivo studies by an inflammatory mediator and not by direct viral infection. Alternatively, some macrophage function relevant to antibacterial processes may be altered in vivo by a direct virus effect, but this particular function was not tested in the assays used in these experiments. For example, Kleinerman and co-workers found that macrophage migration toward chemotactic stimuli was reduced in vivo and in vitro during influenza infection (13). This defect would potentially explain the histological observations in virus-infected animals, since the relative excess of extracellular bacteria and delayed clearance of intracellular bacteria might actually represent decreased influx of new macrophages and excessive ingestion by resident macrophages in the inflammatory foci. As discussed above, this situation could result in delayed intracellular killing. These possibilities have not been investigated in this study but are all testable with available techniques.

In summary, we conclude that in vitro infection of pulmonary macrophages with influenza virus does not impair the two most obviously relevant macrophage functions necessary for bacterial clearance. Our results do not support the hypothesis that defective pulmonary antibacterial defenses observed during influenza infection result directly from infection of resident alveolar macrophages with influenza virus.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant 1-RO1-AI15223-01 from the National Institute of Allergy and Infectious Diseases (to E.L.P.), by American Cancer Society grant PDT-31 (to E.L.P.), and by an American Lung Association training grant (to K.M.N.).

We thank Judy Kaeser for her secretarial assistance and S. T. Donta for reviewing the manuscript.

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