# STUDIES OF CELLULAR DEFENSE AGAINST INFECTION

I. TRANSFORMATION DURING MIXED PNEUMOCOCCAL INFECTION OF MICE

II. ANTIPHAGOCYTIC EFFECT OF INFLUENZA VIRUS ON LEUKOCYTES

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Final Report

by

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FOREWARD

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In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

The information in this report has not been cleared for release to the general public.

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SUMMARY

Part I. The recent demonstration by others of transformation during peritoneal infection of mice by 2 genetically distinct pneumococcal strains supports the notion that transformation may be significant in pneumococcal infection in nature. These studies confirm the occurrence of transformation during mixed infection of mice and define some conditions for its occurrence and its significance.

Mice were inoculated with DNA donor (small type III capsule, low virulence, streptomycin sensitive) and recipient (noncapsulated, low virulence, streptomycin resistant) pneumococci, and the bacteremia in the mice that died was evaluated. Transformants (large type III capsule, virulent, streptomycin resistant) were isolated from up to 80% of mice that died from mixed infection. Transformation occurred in mice that received the donor and the recipient 6 hr apart; hence, active DNA was released and competence developed during growth in vivo. Transformation was detected only with progressive infection by both strains, and then transformants were few in the blood and apparently were not responsible for the death of the animals.

In doubly-infected mice treated with streptomycin, transformation was enhanced; transformants numerically dominated the bacteremia and seemed to cause the death of the mice.

Transformation was also demonstrated for the first time during infection of the respiratory tract.

Part II. Infection of the respiratory tract by influenza virus enhances susceptibility to bacterial infection, e.g., pneumococcal pneumonia.

This synergism has been attributed to (i) destruction of the respiratory epithelium by the virus and (ii) the accumulation of edema in the influenza-infected lungs. In vitro studies by others with guinea pig polymorphonuclear leukocytes (PMN) suggested that a third factor may be important, namely a direct antiphagocytic action of the virus on the cells. These studies were directed at defining further the effect of influenza virus on leukocytes and determining the role of the direct antiphagocytic action of influenza virus in the heightened susceptibility to bacterial infection that accompanies influenza.

The interaction at physiological temperature of influenza viruses with leukocytes from mice, rats, and guinea pigs was complex. Leukocytes differed both by type and species in the way in which they reacted with influenza A and B viruses. Influenza virus attached rapidly to both PMN and macrophages from all 3 species. The outcome of the interaction depended, however, on the relative rates of two competing reactions: (i) receptor destruction by viral neuraminidase followed by elution and (ii) ingestion by the leukocyte of the attached virus followed by intracellular destruction of the virus. Influenza A (WS strain) attached and eluted from both PMN and macrophages of mice and rats and from guinea pig PMN; WS virus was ingested and destroyed by guinea pig macrophages. Influenza B (Lee strain) was ingested and destroyed by both cell types of the 3 species.

Interaction with influenza virus reduced the phagocytosis of pneumococci by exudative PMN and macrophages and by alveolar macrophages. The extent of inhibition depended on the quantity of virus and the duration of virus-cell interaction. There were, however, differences

between species; the phagocytic activity of mouse and guinea pig leukocytes was reduced by virus, but that of rat cells was not.

The effect on phagocytosis was poorly correlated with inhibition by the virus of leukocyte glycolysis and was not solely due to either viral neuraminidase (RDE), i.e., $RDE^+$ and $RDE^-$ strains caused similar changes, or penetration of the cell by the virus.

Although influenza virus can inhibit phagocytosis of pneumococci by guinea pig leukocytes, lung defenses against airborne pneumococci were unaffected in guinea pigs with influenza during that portion of the viral infection in which the virus content of the lung was maximal, but epithelial lesions and edema were minimal. These negative conclusions about the role in vivo of the direct antiphagocytic action of influenza virus further support the thesis that the virus-induced epithelial lesions and edema are primarily responsible for the impaired antibacterial defense of the lungs during influenza.

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# PART I

## TRANSFORMATION DURING MIXED PNEUMOCOCCAL INFECTION OF MICE

Although the recognition of pneumococcal transformation (1) and the identification of the transforming principle as deoxyribonucleic acid (2) were "the first steps" of molecular genetics, the significance of transformation during pneumococcal or other bacterial infections has never been established. Experimentally, transformation has not been demonstrated during infection of the respiratory tract (3), the site of most natural pneumococcal infections, and until recently, pneumococcal transformation was achieved *in vitro* and *in vivo* only with large numbers of heat-killed organisms or DNA extracts as the source of transforming DNA (1, 2, 3, 4). In 1962, however, transformation was recognized among pneumococci growing *in vitro* (5), and a year later, genetic exchange by transformation was demonstrated during peritoneal infection of mice with pneumococci of different genotypes (6). In the latter experiments, the recipient culture was treated to induce competence at the time of injection, and the two strains were inoculated sequentially. Transformants were isolated from the blood of 44% of mice that died of the mixed infection (from 31% of those inoculated), but the role of the transformed pneumococci in the lethal infection was not determined.

The present experiments confirm the occurrence of transformation during mixed pneumococcal infection of mice and define some conditions for its occurrence and its significance.

Materials and methods:

Mice. 18 to 22 gram female mice of the MBR/ICR strain were obtained from Maryland Breeders for Research, Burtonsville, Maryland. The investigators adhered to the "Principles of Laboratory Animal Care" established by the National Society for Medical Research.

Media. Fluid cultures were grown in brain-heart infusion broth (BHI, Difco Laboratories, Detroit, Michigan) containing 10% sterile sheep serum (Pentex Corporation, Kankakee, Ill.). Trypticase Soy agar (BBL, Baltimore, Maryland) with 0.05% sterile sheep blood was the solid medium with or without streptomycin, 500 μg/ml. Tryptose-phosphate broth (Difco) was used for dilutions.

Deoxyribonuclease. Deoxyribonuclease (DNase I, Sigma Chemical Co., St. Louis, Mo.) was always used together with a final concentration of 0.007% $MgSO_4 \cdot 7\ H_2O$.

Streptomycin. Streptomycin sulfate (E. R. Squibb and Sons, New York, N. Y.) was added to blood agar medium in a final concentration of 500 μg/ml and was diluted with sterile water to 25 mg/ml for intramuscular injection into mice.

Mucin. A 6% suspension of granular mucin, type 1701-W (Wilson Laboratories, Chicago, Ill.), was autoclaved and adjusted to pH 7.8 by addition of NaOH.

Pneumococci. The parental strains of pneumococci were kindly provided by Dr. Elena Ottolenghi; their derivation and properties have been described in detail (6), and pertinent characteristics are summarized in Table 1. Stocks were stored anaerobically in defibrinated rabbit blood at 4° C.

Table 1

Properties of the pneumococcal strains IIISIR6, R36NCSm$^r$, and IIISR36NCSm$^r$.

| Strain | Type of capsule | Susceptibility to streptomycin (500 µg/ml) | Colonial morphology | Mouse intraperitoneal $LD_{50}$ (organisms) |
|---|---|---|---|---|
| IIISIR6 | III | Sensitive | Smooth | $10^{6.6}$ |
| R36NCSm$^r$ | — | Resistant | Rough | $10^{8.2}$ |
| IIISR36NCSm$^r$ | III | Resistant | Mucoid | $10^0$ to $10^1$ |

The transformant, IIISR36NCSm$^r$, derived by transformation of the R36NCSm$^r$ with DNA from IIISIR6, was resistant to streptomycin (>500 µg/ml), had a large type III capsule, and was highly virulent for mice, Table 1. Colonies on both plain and streptomycin blood agar were large, mucoid, and easily distinguished from those of the donor and recipient strains.

Preparation of inocula. Cultures for injection into mice were grown in BHI-serum for 4 hr at 37° (the inoculum was 10% by volume of a culture started from stock and grown for 16 hr in BHI-serum) and diluted as required with BHI. Microscopic examination of 4 hr cultures showed a predominance of diplococci and only occasional short chains. Pneumococci were enumerated as colony-forming units on blood agar.

When necessary, organisms were concentrated by centrifugation at 1900 x g for 20 min at 4° and resuspension in BHI to 10% of the original volume.

Growth medium was separated from cells by centrifugation of the

culture at 1900 x g for 20 min at 4° followed by filtration through ultrafine sintered glass (pore size = 1.2 u). Such filtrates contained less than 0.001% of the original number of pneumococci.

When indicated, DNase was added to both recipient and donor cultures immediately prior to inoculation of mice.

For intrabronchial inoculation, the organisms were mixed in 6% mucin immediately prior to inoculation.

Animal inoculation. Donor and recipient cultures (0.5 ml each) were injected separately; except as noted, each animal received the donor immediately after the recipient. Intrabronchial inoculation was done as described by Harford et al. (7); 0.05 ml was inoculated. Mice were exposed to pneumococcal aerosols in a Henderson apparatus equipped with a Collison generator (8). Samples of aerosol were collected in impingers, $AGI_{30}$ (9), containing tryptose-phosphate broth, and the number of colony-forming units on blood agar was determined. Antifoam A (Dow-Corning Corp., Midland, Mich.) was added to both the bacterial suspension in the generator and the impinger fluid; pneumococcal viability was not impaired by the Antifoam A. The product of (i) the duration of exposure, (ii) the respiratory minute volume (10), and (iii) the concentration of viable organisms was taken as the inhaled dose.

Determination of virulence. Mice were inoculated intraperitoneally with 0.5 ml of serial 10-fold dilutions of culture, 10 mice per dilution, and observed for 10 days. The lethal dose 50% ($LD_{50}$) was calculated by the method of Reed and Muench (11).

Examination of mice. Because of the great virulence of the transformed pneumococcus under study here, Table 1, formation of a few, or perhaps only one, transformants within the mouse would initiate a lethal infection. Therefore, only mice that died during the experiments were examined. Within 4 hr of death, the thorax was opened aseptically, and the heart was incised. With a calibrated loop, 0.01 ml of blood was transferred to 1 ml of TP broth. After mixing, 0.1 ml of the broth, or dilutions thereof, was spread on both blood agar and streptomycin blood agar. The nature of the bacteremia was determined by the types and the numbers of colonies on these two plates after 24 hr incubation at 37°. To detect small numbers of transformants in the blood, 0.01 ml of heart blood was inoculated into 5 ml of BHI-serum containing 50 μg of DNase. DNase was included to prevent transformation during in vitro growth; transformants were not detected in the BHI-serum-DNase broth after growth from stock of a mixed culture of donor and recipient bacteria. After time for growth at 37°, a loopful from the BHI-serum-DNase culture was streaked on streptomycin blood agar; the plate was examined after incubation at 37° for 24 hr.

Transformants were recognized by both colonial morphology and growth in the presence of streptomycin. The presence of type III capsule was confirmed by "quellung" with rabbit anti-IIIS serum. The mouse virulence was determined with organisms derived from at least one typical colony of transformant in each type of experiment.

The efficiency of detection of transformants in the blood was determined by examination of defined mixtures of the three pneumococcal

strains, mixtures that reproduced the concentrations of pneumococci in the blood of experimental mice, i.e., $10^7$ pneumococci/ml (see below). Recovery of the transformant approximated predictions from the Poisson distribution, e.g., with 500 and with 20 IIISR36NCSm$^r$ per ml of mixture, these organisms were demonstrated in 90% and 30% of 0.01 ml loopfuls respectively.

In some experiments, sites of inoculation were cultured in addition to the blood. The site, peritoneal or subcutaneous, was opened aseptically and without gross contamination by blood. The samples were obtained with a loop and were streaked on blood agar and streptomycin blood agar plates and placed in BHI-serum-DNase. Colonies were evaluated as described above.

Results:

Transformation during mixed pneumococcal peritonitis. Virulent, streptomycin-resistant pneumococci were recovered from the blood of mice that died after intraperitoneal inoculation of two relatively avirulent strains, IIISIR6 and the streptomycin-resistant R36NCSm$^r$, Table 2. Because it was not found in mice that died of infection with either IIISIR6 or R36NCSm$^r$, the new organism was presumed to have originated by genetic exchange *in vivo* and was designated IIISR36NCSm$^r$. The IIISR36NCSm$^r$ organisms were not found in mice that died after intraperitoneal injection of IIISIR6 and R36NCSm$^r$ plus 20 μg of DNase, Table 2. The effectiveness of DNase is good evidence that the IIISR36NCSm$^r$ arose by DNA-mediated transformation. Transformants were not recovered from mice inoculated simultaneously with R36NCSm$^r$ and the relatively cell-free filtrate of a IIISIR6 culture, but were found

Table 2

Transformation during mixed peritoneal infection and the effect of intraperitoneal DNase.

| IP inoculum | | | | Number of mice | | |
|---|---|---|---|---|---|---|
| IIISIR6 | R36NCSm$^r$ | DNase | No. of experiments | Inoculated | Dead | With transformant |
| $10^8$ | $10^{8.5}$ | 0 | 5 | 47 | 47 | 38 |
| $10^8$ | $10^{8.5}$ | 20 µg | 3 | 30 | 28 | 0 |

in animals inoculated with R36NCSm$^r$ and IIISIR6 cells separated from culture filtrate, Table 3. Hence, transforming DNA was liberated by the donor

Table 3

The role of IIISIR6 culture components as DNA source for transformation *in vivo*.

| IIISIR6* | | Number of mice | | |
|---|---|---|---|---|
| Component | No. of organisms | Inoculated | Dead | With transformant |
| Culture | $10^{8.8}$ | 6 | 6 | 6 |
| Filtrate | $10^{3.5}$ | 10 | 7 | 0 |
| Cells | $10^{8.3}$ | 10 | 10 | 8 |

* 0.5 ml intraperitoneally; all mice received $10^{8.6}$ R36NCSm$^r$ in addition.

pneumococci *in vivo* and not merely injected as a part of the culture components after *in vitro* growth. Taken together, the above experimental and control

observations confirm the previous report of transformation among living pneumococci in mice (6).

In these initial experiments both parental types were recovered from the blood of 51 of the 52 mice in which the transformant was found. Analysis of the mixed bacteremia indicated that the transformant played little role in the death of these mice. The concentration of pneumococci in the blood was approximately $10^7$ organisms/ml, but transformants rarely exceeded $10^5$ organisms/ml or 1% of the total bacteremia. In contrast, mice dead from infection with the transformant alone had bacteremias of about $10^7$ organisms/ml.

The time from inoculation to death and the type of bacteremia varied with the intraperitoneal doses of the parental types, Figs. 1 and 2. In the experiments depicted in Fig. 1, all mice received $10^8$ IIISIR6, a dose sufficient to kill most animals. Mice that received, in addition, a low dose of R36NCSm$^r$ ($10^6$ organisms) died relatively late, and only IIISIR6 was recovered from the blood of most animals. As the dose of R36NCSm$^r$ increased (the dose of IIISIR6 was constant, $10^8$ organisms), the time from inoculation to death decreased. Mixed bacteremia, i.e., IIISIR6 and R36NCSm$^r$ with or without IIISR36NCSm$^r$, was usual with doses of R36NCSm$^r$ between $10^{7.5}$ and $10^9$. Transformation also varied in frequency at different doses of R36NCSm$^r$, occurring in 80% of dead mice at a R36NCSm$^r$ dose of $10^{8.5}$ organisms. At a still higher dose of $10^{9.5}$ R36NCSm$^r$ (in addition to $10^8$ IIISIR6), only R36NCSm$^r$ was recovered from the blood of most mice; samples from the peritoneal cavity also contained only the R36NCSm$^r$. These results indicated that in the case of intraperitoneal inoculation

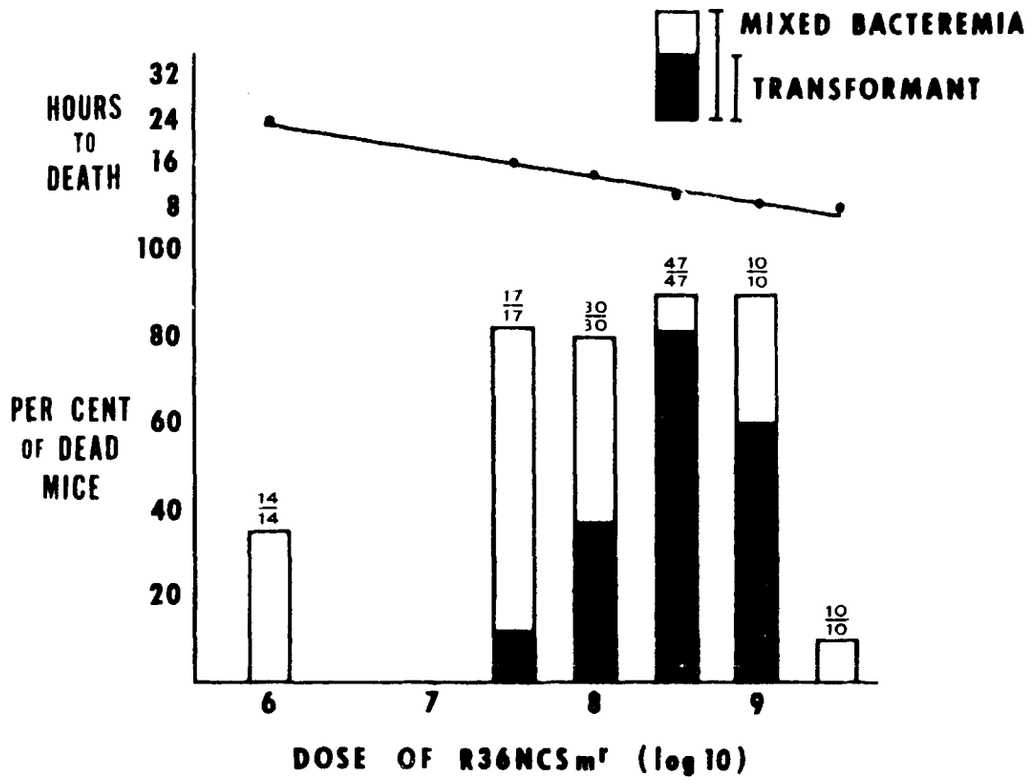


Fig. 1. The relation between the intraperitoneal dose of R36NCSm$^r$, the mean time from inoculation to death, and the nature of bacteremia at death. All mice received $10^8$ IIISIR6 intraperitoneally in addition to the R36NCSm$^r$. Numbers above the bars indicate the number of mice that died/the number injected. Note: All mice that died had pneumococcal bacteremia by at least one strain; the open bars indicate the percent of dead mice from whose blood both donor and recipient were recovered, and the closed bars indicate the percent of dead mice from whose blood transformants were also recovered.

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of the high dose of R36NCSm$^r$ and $10^8$ IIISIR6, the IIISIR6 did not multiply significantly even within the peritoneum.

Because transformation was demonstrated only in mice with a mixed donor and recipient bacteremia, progressive infection by both strains appeared necessary for transformation to be detected under the conditions

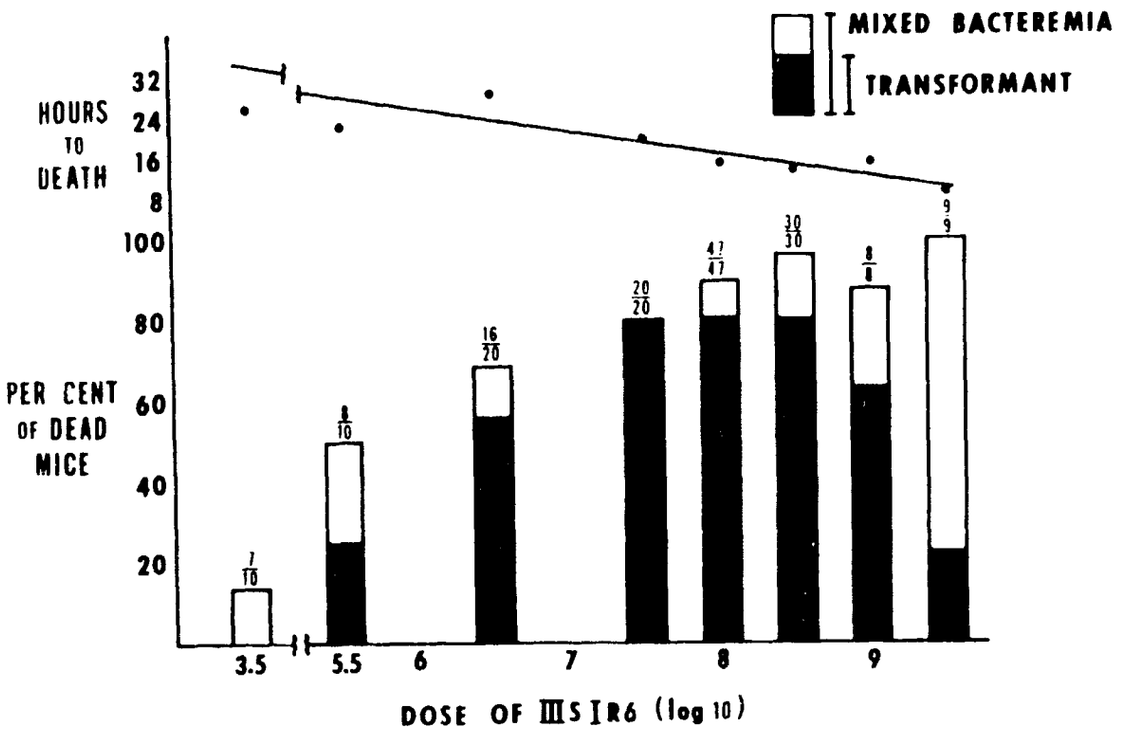


Fig. 2. The relation between the intraperitoneal dose of IIISIR6, the mean time from inoculation to death, and the nature of bacteremia at death. All mice received $10^{8.5}$ R36NCSm$^r$ intraperitoneally in addition to the IIISIR6. Numbers above the bars indicate the number of mice that died/ the number injected. See the Note in the legend for Fig. 1.

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of these experiments. Other factors were operative, however, as the incidence of transformation varied differently with R36NCSm$^r$ dose than did the frequency of mixed donor and recipient bacteremia. For example, in Fig. 1 compare the results with R36NCSm$^r$ doses of $10^{7.5}$ and of $10^{8.5}$, doses that, together with $10^8$ IIISIR6, caused a similar incidence of mixed bacteremia but different frequencies of transformant bacteremia.

The results were somewhat different when the experiment was reversed,

i.e., a constant dose of $10^{8.5}$ R36NCSm$^r$ organisms (sufficient alone to kill most mice) plus varied doses of IIISIR6 (Fig. 2). The time from inoculation to death decreased as the dose of IIISIR6 increased. Both mixed bacteremia and transformation were frequent over a relatively broad range of doses of IIISIR6, $10^{6.5}$ to $10^{9}$ (plus $10^{8.5}$ R36NCSm$^r$). Moreover, only at the highest dose was there marked divergence between the incidence of transformation and of mixed bacteremia. As in the former experiments, transformation was demonstrated only in mice with a mixed bacteremia. These results, together with those above, suggest that active donor infection in the site of infection by recipient organisms was a sufficient condition for transformation. In other words, the dose of donor-organism had to be large enough to initiate active infection. The lower incidence of transformation at the highest dose of IIISIR6 may have been due to early death, i.e., the time between transformation and death was too short in many mice for the IIISR36NCSm$^r$ to multiply and invade the blood in sufficient numbers to be detected. Transformants were not detected, however, in samples of peritoneal exudate from such mice.

In the experiments of Ottolenghi and MacLeod (6), the recipient culture was manipulated so as to be at peak competence at the time of inoculation. In the present experiments, no effort was made to induce competence prior to injection, but the competence of the recipient inoculum <u>per se</u> was not determined. However, the requirement for progressive infection by the recipient suggested that the recipient

organisms became competent during *in vivo* growth. This suggestion was further supported by the results of studies in which the intraperitoneal injections of recipient and donor organisms were separated by time, Table 4. When the dose of recipient was large enough to cause progressive

Table 4

Pneumococcal transformation in mice that received intraperitoneal donor and recipient organisms 6 hr apart.

| First inoculum | Second inoculum | Number of mice | | |
|---|---|---|---|---|
| | | Inoculated | Dead | With transformant |
| R36NCSm$^r$ ($10^{8.6}$) | IIISIR6 ($10^{6.5}$) | 8 | 2 | 1 |
| IIISIR6 ($10^6$) | R36NCSm$^r$ ($10^8$) | 7 | 7 | 2 |

infection, but not sufficient to kill in less than 12 hr, transformants were found in mice that received the donor 6 hr after the recipient. Transformation was not detected after lesser doses of recipient, numbers of organisms that were insufficient to establish active infection prior to the inoculation of the donor organisms. Similar results were obtained when the order of injection was reversed, i.e., donor 6 hr before recipient, Table 4. Hence, both competence of the recipient strain and release of transforming DNA by the donor strain occurred during multiplication in the mouse.

Transformation during pneumococcal infections initiated at separate sites.

The above studies involved injection of both parental strains into the peritoneum, but a common initial site of infection was not essential for transformation in vivo, Table 5. Transformants were recovered from

Table 5

Pneumococcal transformation during infection of mice by donor and recipient organisms introduced at separate sites.

| Subcutaneous (organisms) | Intraperitoneal (organisms) | DNase | Number of mice: Inoculated | Dead | With transformant |
|---|---|---|---|---|---|
| IIISIR6 ($10^{7.3}$) | R36NCSm$^r$ ($10^{8.5}$) | 0 | 16 | 14 | 7 |
| IIISIR6 ($10^{7.3}$) | R36NCSm$^r$ ($10^{8.5}$) | 20 µg | 10 | 5 | 0 |

the blood of 50% of mice that died after receiving the donor strain subcutaneously on the back and the recipient organisms intraperitoneally. These mice had a mixed bacteremia, i.e., donor, recipient, and transformant, and peritoneal fluid contained donor and transformant in addition to the recipient. No transformants were recovered from the subcutaneous site of inoculation. Intraperitoneal DNase prevented transformation, Table 5. Collectively, these results indicate that transformation occurred during a mixed peritoneal infection that resulted from the seeding of the R36NCSm$^r$ peritonitis by the blood-borne IIISIR6.

Transformation during mixed pneumococcal pneumonia. Transformation also occurred during mixed pneumococcal infection in the lungs. In the initial

experiments, pneumonia was produced in mice by intrabronchial inoculation of a mixture of $10^{7.6}$ R36NCSm$^r$ and $10^{6.6}$ IIISIR6 suspended in mucin; 8 of 12 mice died between 24 and 48 hr after inoculation. Transformants were recovered from the blood of 3 of the dead mice. Although one, or both, of the parental strains was also recovered, the transformant numerically dominated the bacteremia in 2 of the 3 mice. The significance of the transformed pneumococci in the death of these mice contrasted with the minor role that they played in mice with mixed peritonitis. Because the donor and recipient organisms were mixed in mucin before inoculation, transformation could have occurred in vitro rather than in the respiratory tract. This seemed unlikely because transformed organisms were not detected in the mixture after completion of mouse inoculation; 10 samples of 0.1 ml, twice the mouse inoculum, were cultured in BHI-serum-DNase, and only the two parental types were recovered.

Unequivocal evidence of transformation after inoculation of donor and recipient strains into the lung was obtained in experiments in which mice received $10^{7.6}$ R36NCSm$^r$ in mucin intrabronchially and 4 hrs later inhaled approximately $10^{5.6}$ IIISIR6 as a small-particle aerosol. Nine of ten mice died of pneumonia and bacteremia within 5 days of exposure, and 3 of them had transformants in the blood.

Effect of streptomycin therapy during mixed pneumococcal peritonitis. Because streptomycin treatment of the DNA donor in vitro enhances transformation (5), the effect of streptomycin therapy on the incidence of transformation in doubly-infected mice was determined, Table 6. The mice received intraperitoneally $10^8$ R36NCSm$^r$ and $10^8$ IIISIR6, a combination that produced

Table 6

The effect of streptomycin therapy on the occurrence of transformation during mixed pneumococcal peritonitis in mice.

| Streptomycin* (mg) | DNase** (μg) | Number of mice | | |
|---|---|---|---|---|
| | | Inoculated*** | Dead | With transformant |
| 0 | 0 | 16 | 16 | 4 |
| 2.5 | 0 | 24 | 19 | 17 |
| 2.5 | 100 | 18 | 8 | 0 |

* Intramuscularly 4 hr after infection.

** Intraperitoneally, 50 μg at the time of infection and 50 μg at the time of streptomycin administration.

*** Mice received $10^8$ R36NCSm$^r$ and $10^8$ IIISIR6 intraperitoneally.

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a mixed donor and recipient bacteremia in most animals, but led to transformation in only a few, Table 6 (see also Fig. 1). Transformants were only a small fraction of the total bacteremia in these mice. Similar mice treated with 2.5 mg of streptomycin intramuscularly 4 hr after infection also had a high mortality (Table 6), but deaths occurred about 20 hr later, i.e., after about 14 hr in untreated mice and 34 hr in treated mice. IIISR36NCSm$^r$ organisms were recovered in 17 (89%) of the 19 dead, treated mice. The IIISR36NCSm$^r$ dominated the bacteremia in each of these 17 mice and often was the only organism isolated. IIISR36NCSm$^r$ was not detected in the blood of streptomycin-treated mice that also received intraperitoneal DNase, Table 6. Hence, the new organism almost certainly arose by transformation and not as a result of selection of mutants by a selective factor,

i.e. streptomycin. Additional studies with DNase suggested that streptomycin treatment actually increased the incidence of transformation rather than merely prolonged life sufficiently to allow detection of transformations that were obscured in the untreated mice because of early death due to mixed donor-recipient bacteremia. With smaller quantities of DNase, e.g., 20 μg as used in earlier experiments, transformation occurred in some streptomycin-treated mice. Hence, streptomycin killing of donor pneumococci *in vivo* released more effective amounts of DNA than were released during *in vivo* growth. Transformation also occurred if the injection of DNase was omitted at the time of streptomycin therapy, again suggesting that streptomycin killing liberated large amounts of DNA. Therefore, the enhancement of transformation by streptomycin treatment of donor cells previously described *in vitro* (5) occurred *in vivo* also. Under these circumstances, therapy had little effect (79% vs. 100% mortality, Table 6) because of the high incidence of transformation that yielded virulent, streptomycin-resistant organisms. The potential of the treatment, i.e., reduction of mortality to below 50%, was only realized when transformation was prevented by DNase administration (Table 6).

## Discussion

The present experiments confirm the earlier demonstration (6) of the transformation of pneumococci during mixed infections in mice and define some conditions necessary for such *in vivo* transformations. After infection by two relatively avirulent strains, one resistant to streptomycin, a new strain was recovered from the blood of dead mice; the new strain was

both streptomycin resistant and highly virulent and was, therefore, clearly different from both parental strains. The exchange involved transfer of capsular genome from the minimally encapsulated (type III) donor to the rough recipient (streptomycin resistant). It is improbable that the new strain arose by mutation. First, the pertinent genotypes of both donor and recipient are stable (6). Secondly, it is unlikely that the mutations required of either the donor or the recipient strains to produce the new strain could occur at the frequency at which the new strain was isolated in these experiments. Finally, the complete prevention of the appearance of the new strain in doubly-infected mice by DNase treatment was clear evidence of genetic exchange by transformation; mutation and other known mechanisms of exchange would not be affected by this enzyme.

It is concluded that the transformation occurred in vivo because the donor and the recipient inocula were kept apart until inoculated and the method employed precluded transformation during the in vitro processing of samples from the mice (see Materials and Methods).

In these studies, the in vivo transformation occurred when two conditions were fulfilled: (i) juxtaposition of donor and recipient and (ii) progressive infection by both strains. Although earlier work failed to demonstrate transformation in the respiratory tract (3), transformation occurred in the present studies when double infection was established. In the earlier studies, progressive infection by the recipient may not have been induced. Clearly the donor and recipient infections do not have to originate either simultaneously or in the same location since donor and

recipient infections initiated 6 hr apart yielded transformation and donor and recipient infections initiated in separate sites produced transformation. In the latter case, a donor-strain bacteremia was established that seeded the site of recipient infection, and transformation occurred in the mixed infection.

Despite juxtaposition at the time of inoculation of the parental strains, transformation did not occur unless both donor and recipient organisms multiplied. The ability to donate transforming DNA is apparently eliminated when the bacterial cell is destroyed by phagocytosis and intraleukocytic lysis, the principal mechanism of host defense. The consequences of the recipient's failure to persist are obvious.

Additional, unrecognized factors probably influence the occurrence of transformation in mixed pneumococcal infection. Even when progressive infection by the parental strains and juxtaposition were both established, the incidence of transformation varied when different doses of donor and recipient were employed to initiate infection (see Figs. 1 and 2). Probably the timing of competence and of release of transforming DNA in a mixed infection, and hence the frequency of transformation, is very sensitive to the ratio of donor and recipient present in the site.

Although the transformed pneumococci had a survival advantage in the host by virtue of their large capsule, the transformant played little role in the death of mice with the mixed peritoneal infection. Once the peritoneal infection was established by both parental strains, bacteremia quickly developed, and the mice died regardless of the presence or absence

of the more virulent transformant. In the pulmonary infections, however, the transformants played a prominent role in the lethal outcome. The relatively avirulent donor and recipient organisms were restricted by the superior phagocytic defense of the lungs (12); but the heavily IIIS encapsulated, phagocytosis-resistant transformant was better able to survive and eventually to invade the blood stream.

The transformed organism was also a major factor in the death of doubly-infected mice treated with streptomycin. The drug terminated the donor infection, and when transformation was prevented by DNase administration, over half the mice cleared the recipient infection and survived. When transformation was allowed to occur, however, most of the mice died, and death was due primarily to the highly virulent, streptomycin resistant transformant.

The role of transformation in pneumococcal infection in nature remains unknown. Both pneumococci and streptococci are frequently abundant in the upper respiratory tract of man, and these organisms are capable of both intra- and inter-species exchange of genetic determinants of virulence and antibiotic susceptibility (1-6, 13-17). Therefore, the natural setting exists in both health and disease for transformation to occur. For example, in a patient with pneumococcal pneumonia, other pneumococci or streptococci could enter the lesion from the nasopharyngeal flora of either the patient or another person and participate in transformation. The emergence of a new organism with totally different antigenic properties or antibiotic susceptibilities might dramatically alter the course of the

disease. The present experiments illustrate this possiblity. Finally, if pneumococcal transformation occurs in nature, the present low level of tetracycline-resistant pneumococci (18-20) may eventually increase.

Conclusions:

Transformant pneumococci (large type III capsule, virulent, streptomycin resistant) were isolated from up to 80% of mice that died from mixed peritonitis initiated by inoculation with DNA donor (small type III capsule, low virulence, streptomycin sensitive) and recipient (noncapsulated, low virulence, streptomycin resistant) pneumococci. Transformation occurred in mice that received the donor and the recipient 6 hr apart; hence, active DNA was released and competence developed during growth in vivo. Transformation was detected only with progressive infection by both strains, and then transformants were few in the blood and apparently were not responsible for the death of the animals.

In doubly-infected mice treated with streptomycin, transformation was enhanced; transformants numerically dominated the bacteremia and seemed to cause the death of the mice.

Transformation was demonstrated for the first time during infection of the respiratory tract.

# PART II

## ANTIPHAGOCYTIC EFFECT OF INFLUENZA VIRUS ON LEUKOCYTES

Influenza complicated by bacterial pneumonia is probably the best known example of a viral infection increasing the susceptibility of the host to bacterial disease, an example for which there is both clinical (21) and experimental documentation (7, 22-24). Two mechanisms are recognized as important in the heightened susceptibility. First, viral lesions of the respiratory epithelium (21) may impair clearance of bacteria by the muco-ciliary apparatus and facilitate direct invasion by the bacteria; results of experiments in mice, however, led Harford to question the nature and the significance of lesions of the bronchial epithelium (25). Second, lung edema may develop and both promote bacterial growth and indirectly impair phagocytosis by diluting the exudate that forms as an antibacterial defense (7, 22, 26). In vitro studies with guinea pig polymorphonuclear leukocytes (PMN; 27-29) suggest that a third factor may be important, namely a direct antiphagocytic action of the virus on the leukocytes.

The present studies are directed at defining the role of the direct antiphagocytic effect of influenza virus in the enhanced susceptibility to bacterial infection that is associated with influenza. In addition, earlier investigations of virus-PMN interaction (30-33) and of the direct antiphagocytic action of influenza virus on PMN (27-29) have been extended to cells from other species and to other types of leukocytes.

Materials and methods:

Animals. Female mice (18 to 22 gm, MBR/ICR strain) were obtained from Maryland Breeders for Research, Burtonsville, Md. Male rats (180 to 200 gm, CD strain) were obtained from Charles River Breeding Laboratories, Inc., Brookline, Mass. Male albino guinea pigs (300 to 400 gm) were obtained from both John C. Landis, Hagerstown, Md. and Bar F Rabbitry, Perry Hall, Md.

Leukocytes. PMN-rich peritoneal exudates (70 to 85% PMN) were stimulated with sterile starch-aleuronat (34) and macrophage-rich exudates (80 to 90% mononuclear cells) were stimulated with sterile beef infusion broth (BIB; 35); mice, rats, and guinea pigs received 2, 5, and 15 ml respectively. PMN-rich exudates were collected 18 hr after the injection of the stimulus. Macrophage-rich exudates were harvested from mice and rats 48 hr and from guinea pigs 72 hr after the injection of the stimulus. The animals were killed with ether, and the cells were washed from the peritoneum with cold modified Hank's solution (36) containing 0.01% bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) and 0.2% glucose (HBG). The cells were concentrated by centrifugation (230 x g at 4° for 5 min) and washed again with HBG. Grossly bloody exudates were excluded.

Guinea pig blood leukocytes were collected from heparinized blood obtained by cardiac puncture of ether-anesthetized animals. After centrifugation (1900 x g at 4° for 5 min) of the blood, the buffy coat was aspirated and washed 3 times with HBG.

Human blood leukocytes were harvested from 100 ml of venous blood drawn into heparinized plastic syringes. The blood was mixed in plastic

bottles with 25 ml of 6% dextran (Cutter Laboratories, Berkeley, Calif.) containing 750 mg of glucose and 1 mg of heparin and allowed to stand at 22° for 60 min. The upper, fluid phase was aspirated and centrifuged (250 x g at 4° for 5 min). The leukocytes were washed once with HBG.

Alveolar macrophages were washed with cold HBG from the respiratory tract of normal rats and guinea pigs that had been killed with pentobarbital. The cells were concentrated by centrifugation (230 x g at 4° for 5 min) and washed once with HBG.

Serum. Serum was harvested from human venous blood, from blood collected aseptically from the incised axillary vessels of ether-anesthetized mice, and from blood obtained by cardiac puncture of ether-anesthetized rats and guinea pigs. The blood was allowed to clot for 6 hr at 4° and centrifuged (1900 x g at 4° for 10 min). The serum was stored in glass vials at -85°.

The mouse, rat, and guinea pig sera contained neither hemagglutination inhibiting (HI) nor neutralizing antibodies against the viruses that were used in these studies and were free of agglutinating and "quellung" antibody against the pneumococci that were used here. Guinea pig serum contained heat labile inhibitors of NWS and Lee virus, but not of WS virus. The human serum had no demonstrable agglutinating and "quellung" anti-type I pneumococcus antibody, but had a HI titer of 1:40 against 10 hemagglutination (HA) units (see below) of WS virus.

Influenza viruses. Three mouse-adapted influenza viruses were kindly supplied by Robert R. Wagner: type A — WS strain (neuraminidase positive)

and NWS strain (neuraminidase deficient), and type B -- Lee strain (neuraminidase positive). Stocks of chicken allantoic fluid containing each virus were collected from infected 9 to 11 day old embryonated eggs and stored in glass vials at -85°. The virus stocks contained no antibiotics and cultures in thioglycolate broth (BBL, Baltimore, Md.) were negative. The HA and the egg-infectious virus contents of stocks of each virus were: WS -- 2048 to 4096 HA units/ml and $10^8$ to $10^9$ egg infectious dose 50%/ml ($EID_{50}$; see below), NWS -- 256 to 512 HA units/ml and $10^6$ to $10^7$ $EID_{50}$/ml, and Lee -- 512 to 2048 HA units/ml and $10^6$ to $10^{7.5}$ $EID_{50}$/ml.

Viruses were concentrated by centrifugation (56,000 x g for 60 min) and resuspension in 10% of the original volume of supernate. WS virus was separated from infected allantoic fluid (i) by centrifugation (100,000 x g for 60 min) and resuspension to the original volume in Hank's balanced salt solution (HBSS), and (ii) by adsorption to chicken erythrocytes at 4° and elution therefrom at 37° into HBSS. The WS virus content of infected allantoic fluid was reduced to less than 0.01% of the original (i) by 3 centrifugations (100,000 x g for 60 min), and (ii) by 3 adsorptions with chicken erythrocytes at 4°.

Normal allantoic fluid. Normal allantoic fluid (NAF) was obtained from uninfected 9 to 11 day old chicken embryos and was stored in glass vials at -85°. The fluid contained no antibiotics, and cultures in thioglycolate broth were negative.

Virus estimation. HA was measured by a standard technique (37) and expressed as HA units, i.e., the reciprocol of the greatest dilution that agglutinated

0.5% chicken erythrocytes. In experiments in which neuraminidase was used, 2% sodium citrate was added to the 0.85% sodium chloride that was routinely used as diluent for the HA test.

Egg infectious virus was determined in 9 to 11 day old embryonated eggs; the eggs were inoculated intraallantoically with 0.1 ml of 10-fold dilutions of specimen, either 4 or 8 eggs per dilution. The presence of HA in the allantoic fluid 48 hr later was taken as evidence of infection. The $EID_{50}$ was calculated by the method of Reed and Muench (11).

Neuraminidase. Receptor destroying enzyme (RDE; from both Hoechst Pharmaceuticals, Inc., Cincinnati, Ohio and Microbiological Associates, Silver Spring, Md.) was always used together with an equal volume of borate buffer with 0.1% calcium chloride (31).

Pneumococci. Type I, type XXV, and noncapsulated (R36NC) pneumococci were kindly supplied by M. R. Smith. Capsulated strains were reisolated each month from the blood of infected mice to insure maximal virulence. Stocks were stored anaerobically at 4° in defibrinated rabbit blood. Cultures were grown at 37° in BIB with 0.2% glucose and 10% serum [sheep serum (Pentex Corp., Kankakee, Ill.) for R36NC and type I, rabbit serum for type XXV] ; types I and XXV were grown for 4 hr and R36NC was grown for 7 hr (these cultures were inoculated with 10% by volume of a 16 hr culture started from stock).

Phagocytic tests. Phagocytosis was measured in vitro in both "dilute" (38) and "concentrated" (39) suspensions of leukocytes, serum and pneumococci. Briefly, in the "dilute" system, which was used with both type XXV and noncapsulated pneumococci, $1.25 \times 10^8$ leukocytes, $6.25 \times 10^8$ pneumococci

in a volume of 0.05 ml, and 0.5 ml of homologous normal serum were mixed and tumbled end-for-end in a screw-capped glass tube at 12 rpm and 37°. In the "concentrated" system, which was used with type I pneumococci (and rarely with noncapsulated organisms), $2.5 \times 10^8$ leukocytes, $2 \times 10^9$ pneumococci in a volume of 0.025 ml, and 0.1 ml of homologous normal serum were mixed; 0.06 ml of the mixture was spread evenly over (i) a 2 x 2 cm area of a glass slide, and (ii) a 2 x 2 cm area of filter paper (Aloe standard, A. S. Aloe Co., St. Louis, Missouri), and both were incubated in a moist filter paper-lined Petri dish. After 30 min incubation for all tests, the cells were recovered, and the per cent of 400 leukocytes of the type under test that contained at least one pneumococcus was determined from microscopic examination of smears stained with methylene blue.

These tests were highly reproducible, i.e., the mean difference between duplicates was less than 15% of the mean per cent phagocytosis. Therefore, the phagocytic value of "test" cells was always compared with the value of "control" cells tested concurrently. The 95% confidence level was used for the significance of differences; reduction of phagocytosis to less than 85% of that in the concurrent control was significant.

Leukocyte motility. The test for leukocyte motility has been described (40).

Eosin Y uptake. Leukocytes were mixed with 0.001% eosin Y, and wet preparations were examined microscopically.

Leukocyte glycolysis. Leukocyte glycolysis was determined as the amount of lactic acid produced during 90 min of anaerobic metabolism of glucose as

described by Fisher and Ginsberg (41). Lactic acid was determined by the method of Barker and Summerson (42).

Macrophage cultures. Macrophages were maintained in vitro in milk dilution bottles; 2 x $10^7$ cells were added to 15 ml of medium [either lactalbumin hydrolyzate in Earle's salt solution (Grand Island Biological Co., Grand Island, N. Y.) or Eagle's basal medium (Grand Island), both containing 20% calf serum (Grand Island) and 200 units/ml of penicillin and 200 µg/ml of streptomycin] and incubated at 37° in a $CO_2$ incubator.

Cell disruption. Cells were disrupted either sonically for 4 min at 10 kc (DF101 Sonic Oscillator, Raytheon Co., Lexington, Mass.) or by 3 cycles of freezing and thawing. Light microscopy indicated complete cell disruption. Virus titers were not affected by such treatment.

Aerosol exposure. Animals were exposed both to viral and to pneumococcal aerosols in a Henderson apparatus equipped with a Collison generator (8). Air samples were collected in an impinger ($AGI_{30}$; 9) containing tryptose phosphate broth (TP; Difco Laboratories, Detroit, Mich.) and antifoam A (Dow-Corning Corp., Midland, Mich.). Doses inhaled by the animals were estimated by the product of (i) the concentration of microorganisms in the aerosol, (ii) the respiratory minute volume (10), and (iii) the duration of exposure.

Enumeration of organisms in the lungs. Animals were killed with intraperitoneal pentobarbital, and the lungs were removed aseptically, ground in a mortar with sterile sand, and suspended in 10 ml of TP. Pneumococci were enumerated as colony forming units on Trypticase Soy agar (BBL) containing 0.05% sheep blood. Virus was measured as $EID_{50}$ after 3 freeze-thaw cycles.

Results:

Interaction of influenza viruses with exudate leukocytes in vitro. As described by others (30-33), influenza A virus rapidly attached to guinea pig exudate leukocytes at 4°, and to mouse and rat leukocytes as well (Table 7). Although the macrophage-rich exudates adsorbed slightly

Table 7

The attachment of WS virus to exudate leukocytes during 60 min at 4°.

| Cells X $10^7$ | Type of exudate | Supernate HA/Original HA* | | |
|---|---|---|---|---|
| | | Mouse | Rat | Guinea Pig |
| 0.5 | PMN-rich | 0.25 | 0.25 | 0.25 |
| | Macrophage-rich | 0.13 | 0.50 | 0.13 |
| 4.0 | PMN-rich | 0.12 | 0.13 | 0.13 |
| | Macrophage-rich | 0.06 | 0.06 | 0.06 |

* 1024 to 2048 HA units of WS virus.

more virus than the PMN-rich exudates, the amounts of virus adsorbed to both types of exudates were generally similar. Both types of cells, therefore, had receptors. Experiments with influenza B virus yielded similar results. With both viruses and with cells from all three species, the attached virus was completely eluted during incubation at 37° after addition of 500 units of RDE (prewarmed to 37°).

The results were different if the virus and the leukocytes interacted

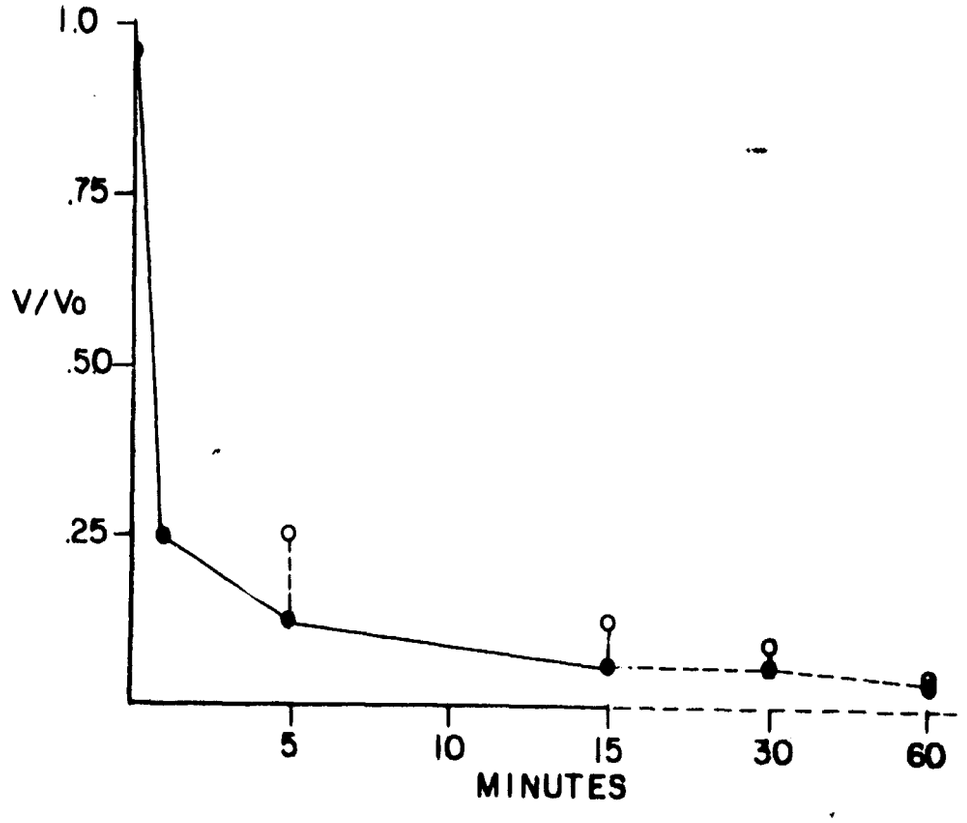


Fig. 3. Adsorption and ingestion of WS virus by guinea pig exudate leukocytes in vitro at 37°. V/Vo is the fraction of the original HA (1024 HA units) that remained in the supernate after incubation with $10^8$ guinea pig leukocytes (PMN-rich). Solid circles indicate supernatant virus after incubation with the cells for the indicated time. Open circles represent the virus in the supernate after the incubation of each set of cells with 500 units of RDE for an additional 30 min at 37°.

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throughout at 37°. As described by Ginsberg and Blackmon (31), WS virus was rapidly adsorbed by the guinea pig leukocytes and did not elute upon continued incubation (Fig. 3). Furthermore, the amount of virus that could be eluted by 500 units of exogenous RDE decreased rapidly (Fig. 3). When

glycolytic metabolism of the cells was inhibited with 0.02 M sodium fluoride, the WS virus attached normally, but eluted during incubation with 500 units of RDE at 37° (spontaneous elution was undoubtedly occurring simultaneously; Table 8). Collectively, these results indicated

Table 8

Inhibition by sodium fluoride of the ingestion of WS virus by guinea pig exudate leukocytes.

| Cells treated with* | HA units | |
|---|---|---|
| | Adsorbed** | Eluted by RDE*** |
| Sodium chloride | 960 | 32 |
| Sodium fluoride | 960 | 512 |

* $10^8$ cells (PMN-rich) plus 0.02 M NaCl or NaF; cells and NaCl or NaF were incubated at 4° for 30 min before virus was added.

** 1024 HA units was added.

*** 500 units of RDE was added to the cells, and they were incubated at 37° for 30 min.

that the WS virus attached to guinea pig exudate leukocytes by a passive process and, at 37°, rapidly entered the cell by a mechanism dependant on active glycolytic metabolism by the cell, presumably phagocytosis.

Ingested virus (HA or infectivity) was not recovered from disrupted cells after incubation at 37° for up to 6 hr, and new virus was not formed in macrophages that were cultured in vitro after ingestion of WS virus.

To determine whether one cell type, or both, in the guinea pig exudate (both were always present in an exudate even though one cell type was numerically dominant) was responsible for ingestion and destruction of WS virus at 37°, WS virus was incubated for 60 min with both PMN-rich and macrophage-rich exudates and with mixtures of the two. After incubation, the cells were recovered by centrifugation (230 x g for 5 min), washed, and sonically disrupted; the virus content (HA and egg infectivity) of the cells and of the combined supernate and wash was determined. Recovery of virus in 0 time samples was from 70 to 100%. The $\log_{10}$ of the fraction of the original HA that was recovered after 60 min decreased linearly with increasing cell number of both PMN-rich and macrophage-rich populations (Fig. 4). (The cell

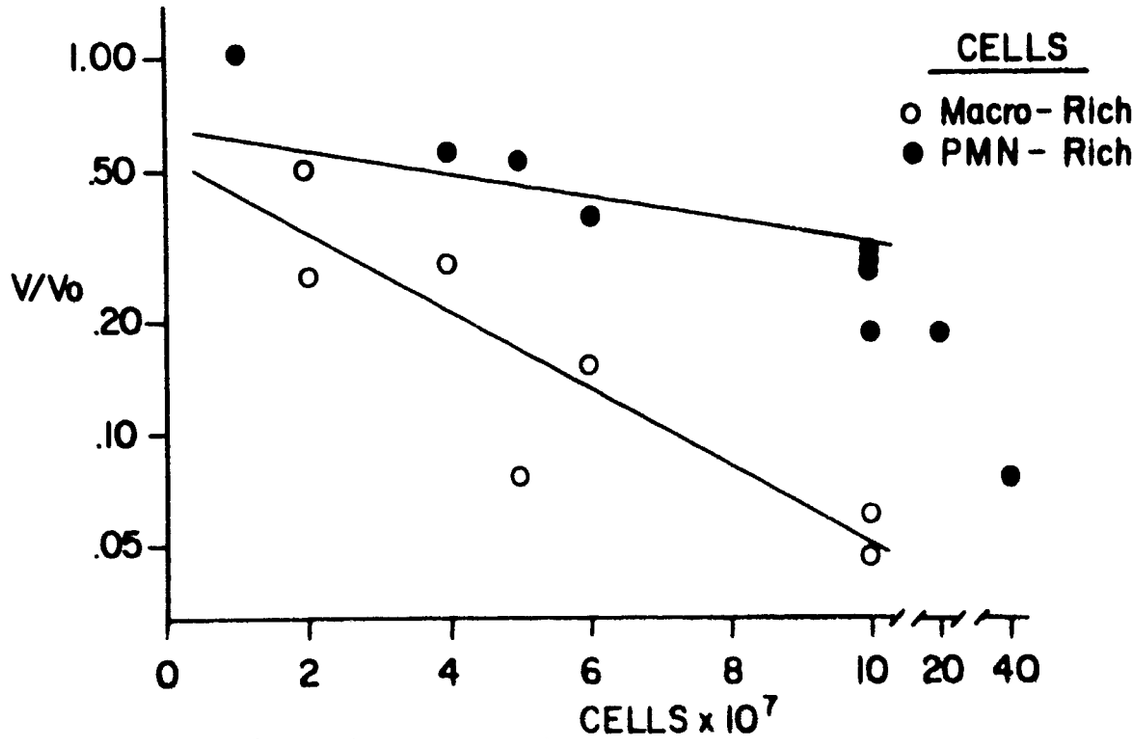


Fig. 4. The relation between the elimination of the hemagglutinin of WS virus and the number of guinea pig leukocytes. See text.

associated virus was relatively constant throughout, and hence, the results obtained by the use of the total virus recovered paralleled those with the supernatant virus alone. The total virus recovery was chosen to depict most completely the entire virus-cell interaction. Furthermore, some of the cell-associated virus probably was unattached virus in the cell button; such virus was accounted for in the sum of the virus recovered in cells and supernatant.) The slope was greatest, however, for the macrophage-rich exudates, thus suggesting that the macrophages were primarily responsible for the ingestion and destruction of virus. This was more evident when the $\log_{10}$ of the fraction of original HA that was recovered was plotted as a function of the number of PMN or of macrophages present in each exudate (Fig. 5); the elimination of virus was clearly

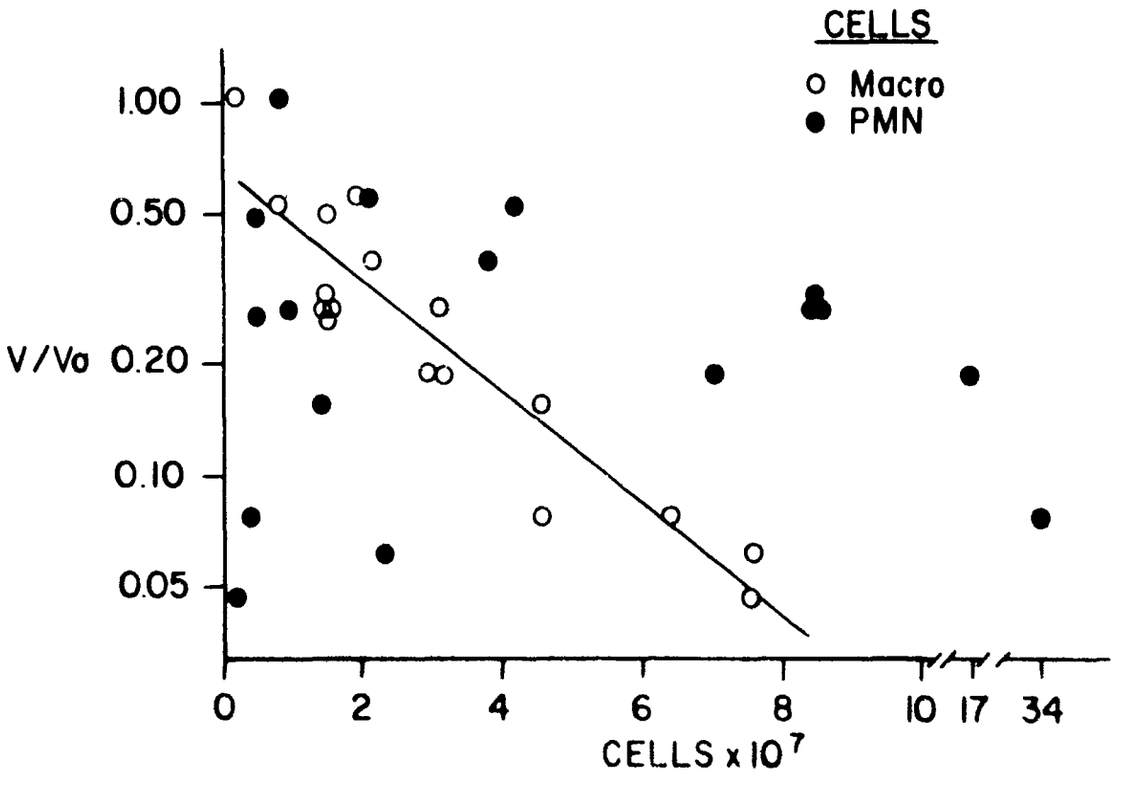


Fig. 5. The relation between the elimination of the hemagglutinin of WS virus, the number of guinea pig macrophages, and the number of guinea pig PMN. See text.

related to the macrophages and independent of the PMN. Virus that attached to PMN, therefore, must have eluted spontaneously at 37°, and the uptake of influenza A virus by PMN-rich exudates (see above) was due to the small portion of macrophages present and not to the PMN. The results of egg infectivity determinations were similar, i.e., both the slope and extent of virus elimination by macrophages were similar regardless of whether HA or egg infectivity was determined. The elimination of virus was real, therefore, and not merely a manifestation of a leukocytic inhibitor of hemagglutination (31).

If NWS virus (neuraminidase-deficient) was incubated at 37° with guinea pig leukocytes, it was ingested (adsorbed and nonelutable by RDE) by PMN as well as by macrophages (Table 9). Although Lee virus is

Table 9

Adsorption and ingestion of NWS and of Lee virus by guinea pig exudate leukocytes during 60 min at 37°.

| Cells X $10^7$ | Type of exudate | HA units | | | |
|---|---|---|---|---|---|
| | | NWS* | | Lee* | |
| | | Adsorbed** | Eluted*** | Adsorbed | Eluted |
| 2 | PMN-rich | 448 | 32 | 866 | 16 |
| | Macrophage-rich | 480 | 8 | 960 | 16 |
| 10 | PMN-rich | 504 | 8 | 1016 | 8 |
| | Macrophage-rich | 504 | 16 | 1016 | 16 |

* Original HA was 512 units.

** Original HA less supernatant HA.

*** HA in the supernate after 37° incubation for 30 min of 500 units of RDE and cells that had adsorbed virus.

neuraminidase positive, it too seemed to enter both PMN and macrophages from guinea pigs at 37° (Table 9).

Exudate leukocytes from mice and from rats were alike in their interaction with WS virus at 37°. The virus rapidly attached to and eluted from both PMN and macrophages (Fig. 6). Afterwards, the cells could not adsorb additional virus at 4°, thus indicating that receptors had been destroyed during the elution process. Both PMN and macrophages of rats ingested NWS virus and Lee virus at 37° (Table 10). Mouse PMN and macrophages adsorbed ($10^8$ cells of either type adsorbed 2[illegible] of 256 HA units in 60 min at 37°) and ingested (only 16 to 32 HA units were eluted in 30 min at 37° by 500 units of RDE from the cells that had adsorbed virus) NWS virus. The main difference, therefore, in the

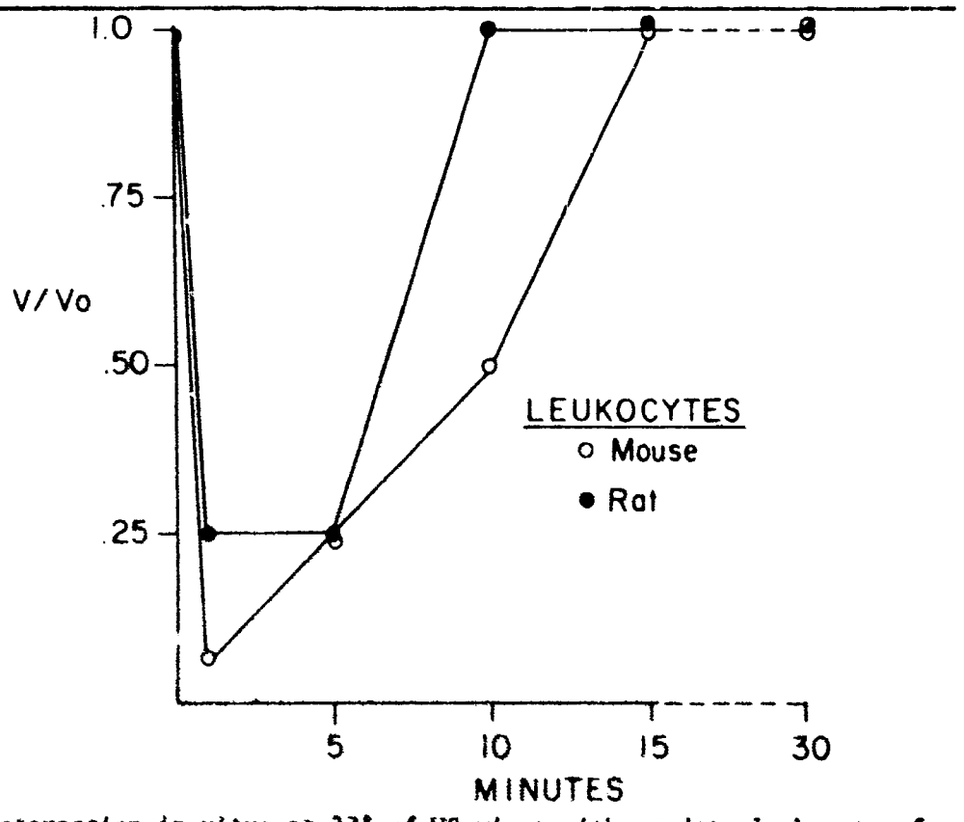


Fig. 6. Interaction in vitro at 37° of WS virus with exudate leukocytes from mice and rats. V/Vo is the fraction of the original HA (1024 or 2048 HA units) that remained in the supernate after incubation with 6 x $10^7$ mouse and $10^8$ rat leukocytes.

Table 10

Adsorption and ingestion of NWS and of Lee virus by rat exudate leukocytes during 60 min at 37°.

| Cells X $10^7$ | Type of exudate | HA units | | | |
|---|---|---|---|---|---|
| | | NWS* | | Lee* | |
| | | Adsorbed** | Eluted*** | Adsorbed | Eluted |
| 2 | PMN-rich | 384 | 32 | 448 | 32 |
| | Macrophage-rich | 384 | 32 | 348 | 32 |
| 10 | PMN-rich | 480 | 32 | 480 | 32 |
| | Macrophage-rich | 480 | 8 | 480 | 32 |

* Original HA was 512 units.

** Original HA less supernatant HA.

*** HA in the supernate after 37° incubation for 30 min of 500 units of HDE and cells that had adsorbed virus.

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interaction of influenza viruses with guinea pig exudate leukocytes and with those from mice and rats was that the guinea pig macrophages ingested WS virus before it could elute, but mouse and rat macrophages did not.

Effect in vitro of influenza viruses on phagocytosis of pneumococci. Leukocytes and virus (or NAF for controls) plus 30% by volume of homologous normal serum were incubated in vitro at 37° for 60 and 120 min respectively for macrophage-rich and PMN-rich exudates. The normal serum was included to preserve cell viability during incubation, but neither was essential for nor enhanced the action of the virus on the leukocytes. After incubation with the virus, the cells were recovered and tested for phagocytosis of pneumococci in vitro. Unless otherwise specified, the

term phagocytosis in this section will refer to phagocytosis of type I pneumococci in the "concentrated" test system.

WS, NWS, and Lee viruses inhibited phagocytosis by both PMN and macrophages from peritoneal exudates of guinea pigs (Fig. 7). WS-infected allantoic fluid from which most virus was removed, either by centrifugation or by adsorption with chicken erythrocytes, did not affect the phagocytic activity of guinea pig PMN (Fig. 8). The separated virus, however, reproduced the effects of the whole fluid (Fig. 8). These results implicated the virus per se in the inhibition of phagocytosis.

The extent of inhibition decreased if either the duration of

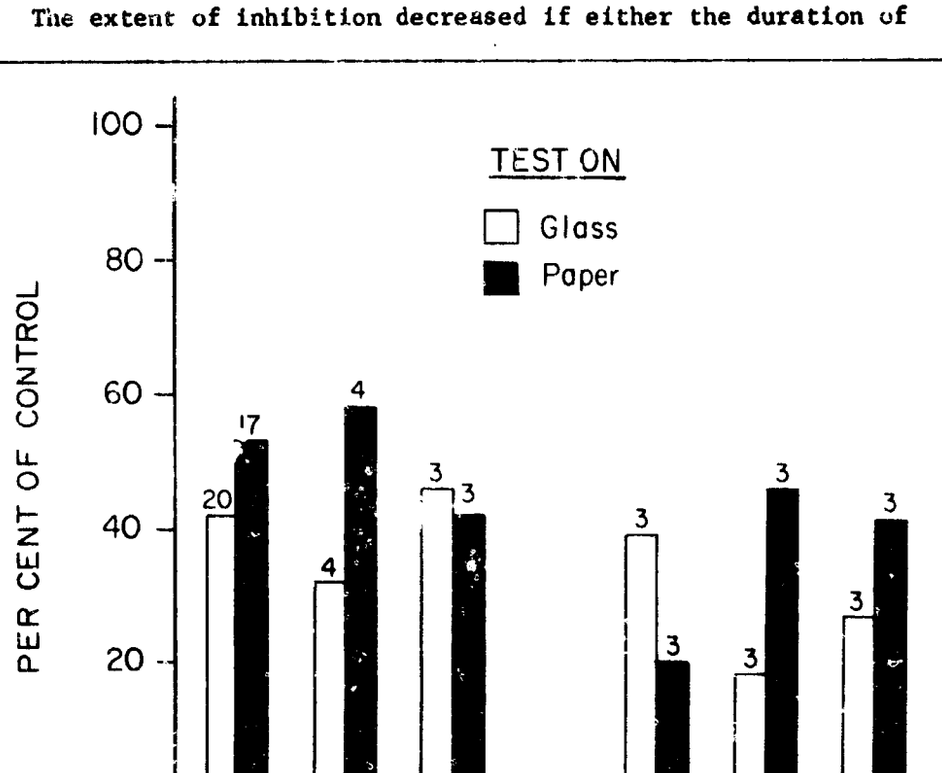


Fig. 7. The effect in vitro of influenza viruses on phagocytosis of pneumococci by guinea pig exudate leukocytes. See text. Means are shown; the number of tests is indicated above each bar. Control cells were incubated with NAF.

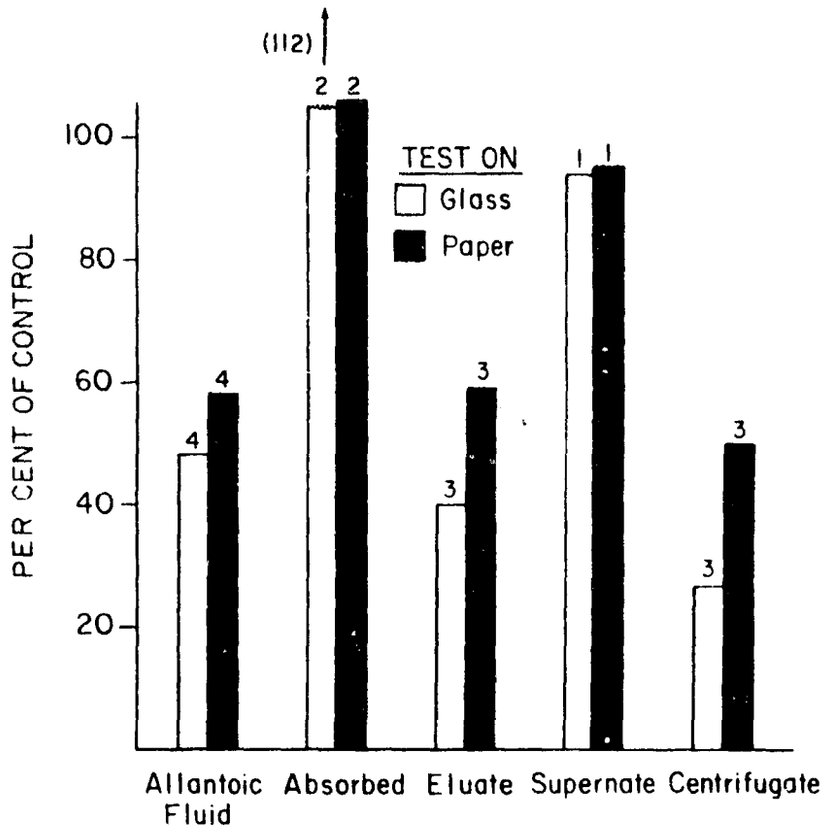


Fig. 8. The association of the antiphagocytic action of WS-virus-infected allantoic fluid with the virus. See text. Means are shown; the number of tests is indicated above each bar. Control cells were incubated with NAF processed in the same way as infected fluid.

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virus-cell incubation (Fig. 9) or the amount of virus (Fig. 10) was reduced. Neither 10-fold concentration of virus (Fig. 10) nor prolongation of virus-cell incubation further reduced phagocytosis.

Phagocytosis by guinea pig PMN of pneumococci other than type I was also inhibited by WS virus, e.g., phagocytosis of noncapsulated organisms was 67% and 60% of control respectively on glass and paper.

The nature of the phagocytic test was crucial in demonstrating the antiphagocytic effect of influenza virus. In the "dilute" system,

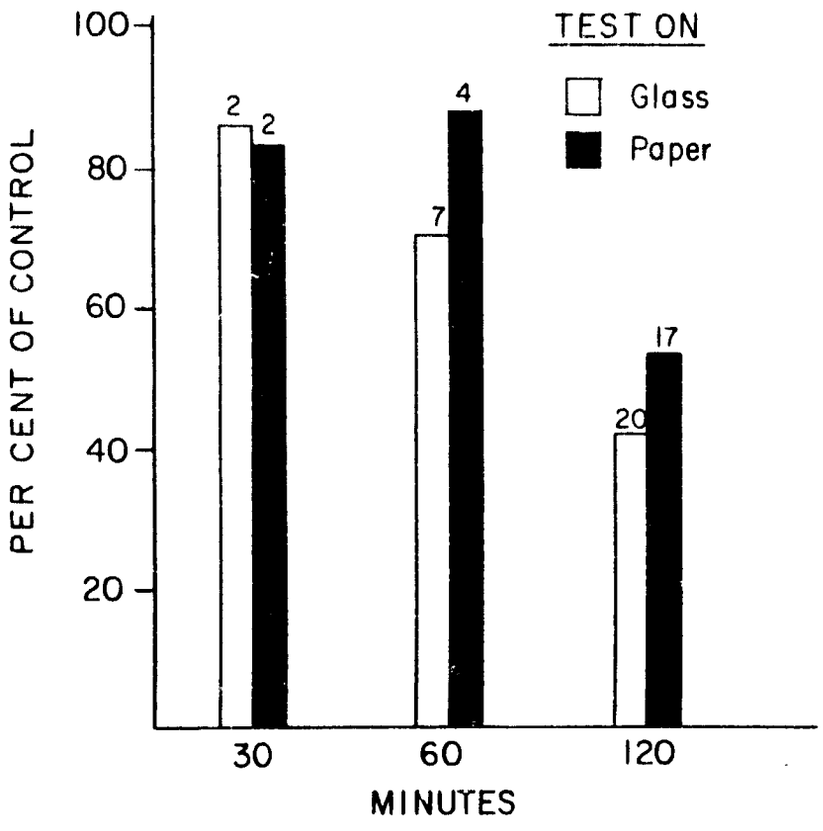


Fig. 9. The relation between the WS virus-cell incubation time and the inhibition of phagocytosis by guinea pig exudate PMN. See text. Means are shown; the number of tests is indicated above each bar. Control cells were incubated with NAF.

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WS virus did not reduce phagocytosis either by guinea pig PMN of noncapsulated (104% of control) or type XXV (94% of control) pneumococci or by guinea pig macrophages of noncapsulated pneumococci (103% of control). NWS and Lee viruses also failed to inhibit phagocytosis by guinea pig leukocytes in the "dilute" system.

WS virus impaired phagocytosis by mouse PMN (30% of control) and macrophages (50% of control) on paper (phagocytosis on glass by control mouse cells was insufficient for inhibition to be detected).

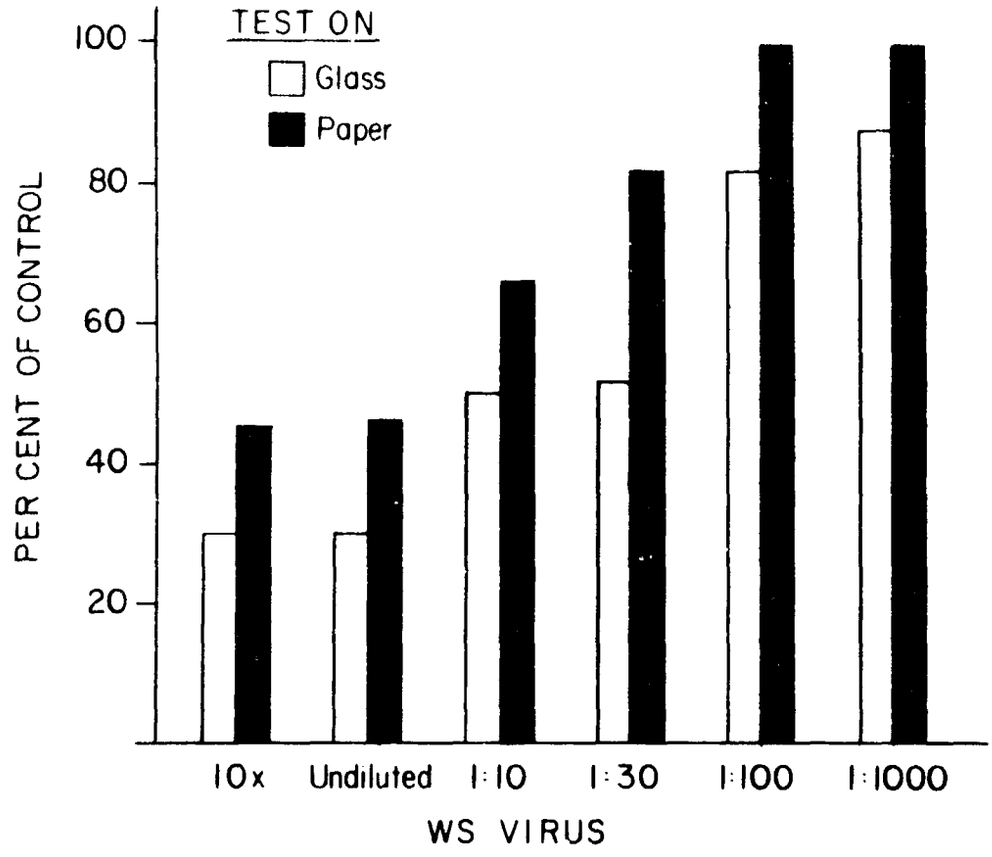


Fig. 10. The relation between the quantity of virus and the inhibition of phagocytosis by guinea pig exudate PMN. Undiluted virus contained $10^{8.5}$ $EID_{50}$/ml. Virus was diluted with NAF. Control cells were incubated with NAF.

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Rat exudate PMN and macrophages were, however, resistant to the antiphagocytic action of influenza viruses as determined in both the "concentrated" system (Fig. 11) and the "dilute" system (not shown). Neither 1 hr extension of virus-cell incubation nor the use of 10-fold concentrated viruses affected the results.

The effect <u>in vitro</u> of WS virus on phagocytosis by alveolar macrophages paralleled that on the leukocytes from peritoneal exudates, i.e., phagocytosis by guinea pig alveolar macrophages was inhibited

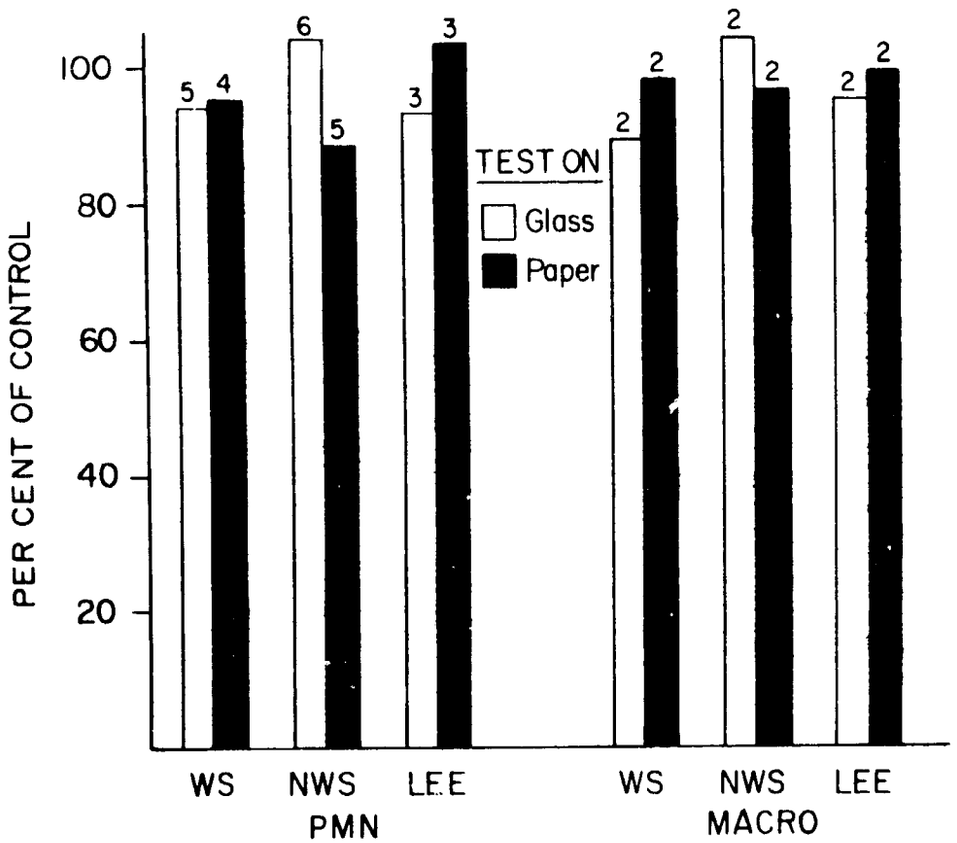


Fig. 11. The failure of influenza viruses in vitro to inhibit phagocytosis of pneumococci by rat exudate leukocytes. Means are shown; the number of tests is indicated above each bar. Control cells were incubated with NAF.

(33% and 60% of control on glass and on paper respectively), but that by rat cells was not (110% and 104% of control on glass and on paper respectively).

Guinea pig blood PMN differed, however, from exudate PMN in susceptibility to the antiphagocytic action of WS virus; they were not inhibited (95% and 107% of control on glass and on paper respectively). This observation adds to the known functional differences between blood and exudate leukocytes, e.g., the former both fail to release leukocytic

pyrogen during incubation in physiological saline (45) and resist the antiphagocytic effects of hyperosmolality (40), but the latter do not.

Human blood leukocytes also resisted the antiphagocytic action of WS virus (119% and 102% of control on glass and on paper respectively). Human exudate leukocytes were not tested.

Effect in vitro of influenza viruses on other properties of leukocytes. Interaction with influenza viruses altered several properties of leukocytes in addition to phagocytosis. Both PMN and macrophages were agglutinated at 37° soon after addition of virus. The agglutination was reversed, however, upon continued incubation at 37°. (The virus-treated leukocytes used for phagocytic tests were not significantly more clumped than control cells that had been incubated with NAF.) Both the motility and the capacity to exclude eosin Y of guinea pig and mouse exudate leukocytes were reduced after interaction with influenza virus *in vitro*. Even if eosin Y uptake is equated with cell death, the increase in the number of leukocytes that were stained by eosin Y after contact with virus was insufficient *per se* to account for the reduction in phagocytosis. Neither the motility nor the staining by eosin Y of rat leukocytes was affected by the influenza viruses. Thus, the susceptibility of leukocytes to the antiphagocytic action of influenza virus paralleled their susceptibility to inhibition by the virus of motility and exclusion of eosin Y.

Ingestion of influenza virus did not lead to degranulation of guinea pig PMN; the number of granules seemed to be the same in cells that had been incubated with NWS and Lee viruses, with NAF, or with HBSS.

Fisher and Ginsberg observed that influenza virus inhibited glycolysis by guinea pig PMN (41); these observations were confirmed (Table 11). The effect on glycolysis was due to the virus for WS-infected

Table 11

The effect of influenza viruses on the glycolysis of exudate leukocytes from guinea pigs and rats.

| Cells incubated* with | Lactic Acid Produced*** (µg) | | | |
|---|---|---|---|---|
| | Guinea Pig | | Rat | |
| | PMN | Macrophages | PMN | Macrophages |
| NAF | 1050 | 845 | 775 | 750 |
| WS | 275 | 630 | 310 | ND |
| NWS | 260 | 825 | 870 | ND |
| Lee | 955 | 535 | ND | ND |
| Conc.** WS | ND+ | 210 | 120 | 610 |
| Conc. NWS | ND | 175 | 205 | 930 |
| Conc. Lee | 625 | 450 | 565 | 680 |

* The cells were incubated with allantoic fluid - 30% homologous normal serum at 37° for 120 min and 60 min with PMN and macrophages respectively.

** 10-fold concentrated

*** Produced by $1.25 \times 10^8$ cells from the anaerobic metabolism of glucose in 90 min.

+ Not done.

allantoic fluid from which most virus was removed, either by centrifugation

or adsorption with chicken erythrocytes, did not affect glycolysis, but the separated virus reproduced the effects of the unaltered infected fluid. Extension of the studies to macrophages and to rat leukocytes indicated, however, that not all leukocytes were alike in their susceptibility to inhibition of glycolysis by influenza viruses (Table 11). For example, glycolysis by rat macrophages was normal after incubation with 10-fold concentrated virus, but that by rat PMN was reduced. Also, more virus was needed to inhibit glycolysis by guinea pig macrophages than by PMN. Finally, the viruses differed in their ability to inhibit glycolysis, e.g., more Lee than NWS was needed to inhibit guinea pig PMN and, even then, the extent of inhibition was less.

Effect in vivo of influenza virus on phagocytosis of pneumococci in vitro. WS virus, or NAF, was injected into preformed peritoneal exudates in living guinea pigs and rats. After 120 min and 60 min with PMN-rich and macrophage-rich exudates respectively, the animals were killed, and the exudates were collected and tested for phagocytosis in vitro. In the in vivo experiments, phagocytosis by guinea pig leukocytes in vitro was not reduced by amounts of virus that were effective in vitro, e.g., $10^{8.5}$ $EID_{50}$ per $2.5 \times 10^8$ cells. With more virus, however, phagocytosis by guinea pig cells was reduced (Fig. 12). Rat leukocytes in vivo resisted the antiphagocytic action of WS virus, at least by amounts of virus greater than those that inhibited guinea pig cells (Fig. 12).

Clearance of pneumococci from the lungs during influenza. Rats and guinea pigs were infected with influenza viruses by exposure to WS virus aerosols;

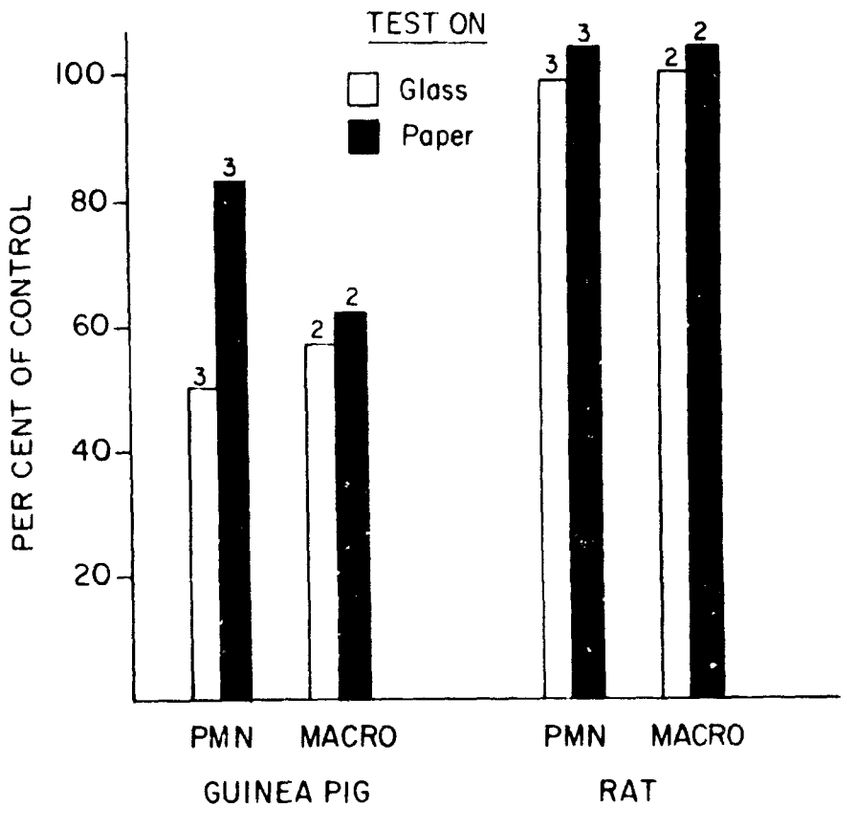


Fig. 12. The effect of WS virus *in vivo* on the phagocytic activity *in vitro* of exudate leukocytes from guinea pigs and rats. See text. Approximately $10^{1.5}$ $EID_{50}$ was injected intraperitoneally per cell in the harvested exudate. Means are shown; the number of tests is indicated above each bar. Control cells were harvested from animals that received NAF intraperitoneally.

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the mean inhaled dose was $10^{5.1}$ $EID_{50}$. In both species, the virus multiplied in the lungs to a peak of approximately $10^{7}$ $EID_{50}$ between 24 and 48 hr after exposure; the amount of virus in the lung declined rapidly during the third day. All animals survived the infection and developed HI antibody. At the time of peak lung virus, the lungs contained no gross lesions, and microscopic lesions were minimal: scattered bronchitis and bronchiolitis.

Small gross lesions, hemorrhagic edema of alveoli, and necrosis of bronchial epithelium did not appear until later.

Forty-eight hr after exposure to WS virus, rats and guinea pigs, together with both normal and NAF-aerosol exposed controls, inhaled airborne type I pneumococci. Immediately and 24 hr after pneumococcal exposure, animals were killed, and the number of pneumococci in the lungs below the tracheal bifurcation was determined (Fig. 13). Additional animals were observed for mortality.

The number of pneumococci was remarkably similar in the lungs of

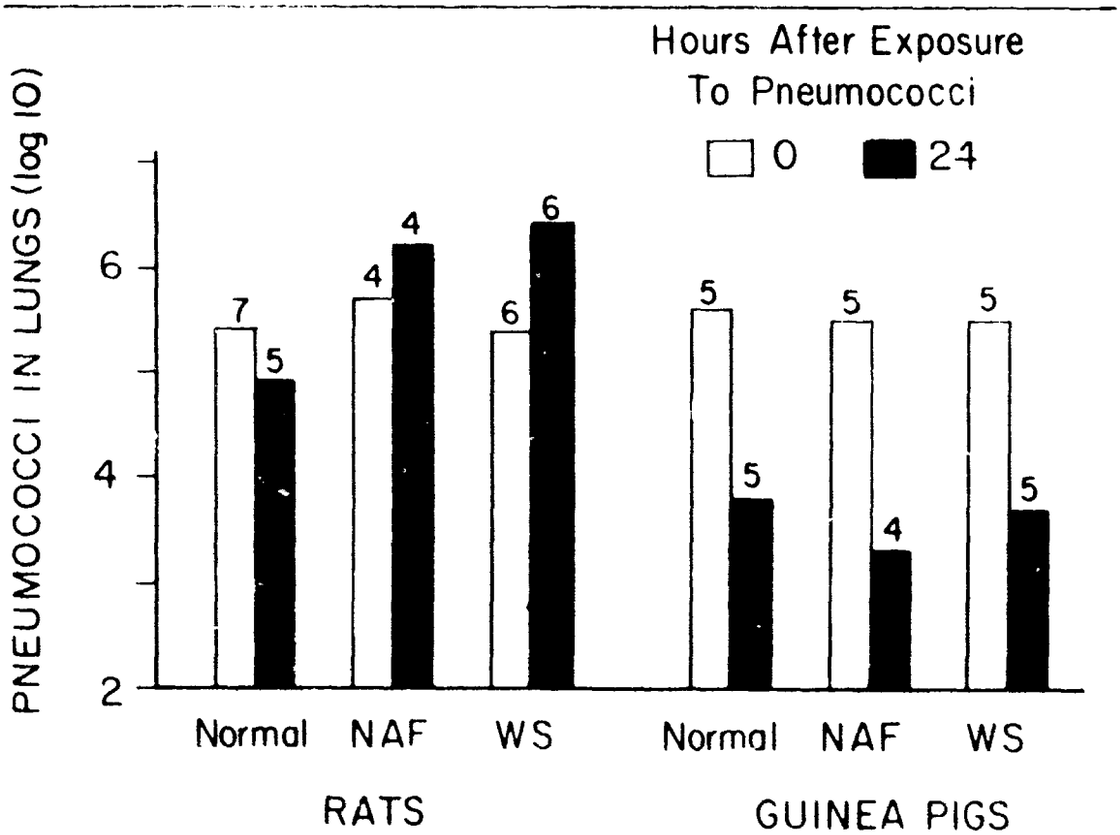


Fig. 13. The survival of type I pneumococci in the lungs of rats and guinea pigs with influenza. See text. Means are shown; the numbers above the bars indicate the number of animals in the group.

all groups immediately after exposure and was compatible with predictions based upon the concentration of pneumococci in the aerosols. In rats, the number of pneumococci was similar or slightly higher 24 hr after exposure, and the number did not differ significantly among the control rats and those with influenza. Mortality from the pneumococcal infection was higher in rats with influenza (42%) than in normal (21%) and NAF-exposed (25%) controls. The excess mortality, however, occurred late, i.e., 9 to 10 days after pneumococcal exposure, and probably reflected the interplay of pulmonary lesions caused by the virus, which did not develop until after the time of the clearance study, and the persisting pneumococci.

In guinea pigs, the number of pneumococci in the lungs decreased during the 24 hr after exposure, but again the number in animals with influenza did not differ from that in control guinea pigs. Mortality was nil in all groups.

Because the amount of virus in the lungs was decreasing during the interval between the estimations of the pneumococcal populations and because the antiphagocytic action of the virus depends on the amount of virus, additional rats and guinea pigs were challenged with airborne pneumococci 24 hr after either infection by airborne WS virus or exposure to NAF aerosols. The interval of study of the pneumococcal populations, therefore, encompassed the period of peak lung virus concentrations. The results were, however, the same, i.e., the number of pneumococci in the lungs 24 hr after pneumococcal exposure was the same in the influenza-infected and in the control rats and guinea pigs.

Discussion:

The interaction of influenza virus with leukocytes is complex and involves at least 4 separate reactions. First, the virus attaches to receptors on the cell surface by a process analogous to attachment to erythrocytes; it is both relatively insensitive to temperature and independent of energy production by the cell, i.e., attachment proceeds nearly as rapidly at 4° as at 37° and with leukocytes inhibited by sodium fluoride. After attachment, 2 reactions compete, (i) the action of viral neuraminidase on the receptors to cause elution of the virus and (ii) the ingestion of attached virus by the leukocyte. The first of these reactions is critically dependent on temperature; the enzyme is essentially inactive at 4° and maximally active at physiological temperature, 37° (46). The second of the competing reactions, ingestion of virus, depends on active cell glycolysis, and hence, is inhibited by both low temperature and enzyme inhibitors such as sodium fluoride. Finally, intraleukocytic virus is apparently destroyed by an unknown mechanism.

The outcome of the virus-leukocyte interaction is, therefore, dependent on the rates of the competing events of elution and ingestion. The results of incubation of WS and of NWS viruses with rat leukocytes at 37° illustrates well the competition. WS virus attaches, and the viral neuraminidase rapidly destroys the receptor site and permits the virus to elute. NWS virus attaches, but because it is neuraminidase-deficient, does not elute and is ingested by the leukocytes. The outcome of the interaction of other viruses with the leukocytes of mice, rats,

and guinea pigs can also be explained on the basis the comparitive rates of elution and ingestion, but the actual rates have not been determined. For example, the rate of receptor destruction by WS virus on both PMN and macrophages of mice and rats and on guinea pig PMN exceeds the rate of ingestion, but guinea pig macrophages ingest the virus more rapidly than receptors are destroyed. There are no data to indicate whether these macrophage receptors are more resistant than those of the other leukocytes to WS neuraminidase or guinea pig macrophages simply ingest the virus more rapidly than the other leukocytes. The results with Lee virus, also, support the hypothesis. Greater destruction of receptors is apparently required for elution of Lee than of WS virus (47); thus, the rate of elution is reduced, and ingestion dominates even though the virus has neuraminidase.

The same considerations of rates of elution and ingestion help to explain the occasional differences between the results of these and of earlier studies (30, 31, 33) that demonstrated the ingestion of influenza viruses by guinea pig PMN. First, a temperature shift during an experiment, e.g., incubation at low temperature to study attachment and then at 37° to study elution (30, 33), may affect the competing reactions, i.e., elution and ingestion, differently and, thereby, yield different results from those obtained in experiments conducted solely at physiological temperature. Second, the addition of influenza immune serum, either before virus cell contact or after attachment at 4° (33), to the virus-cell mixture may favor ingestion; such experiments are principally relevant to

*in vivo* virus-leukocyte interactions in the presence of antibody. Third, because the kinetics of receptor destruction differ among influenza viruses (47), experiments conducted with one do not necessarily provide a basis for generalization about others. Finally, because leukocytes differ both by cell type, i.e., PMN and macrophages, and by species in their capacity to ingest virus and because exudates invariably contain more than one cell type, special techniques are needed to determine whether only one cell type or both are responsible for the outcome of the virus-cell interaction. The present results illustrate well the way in which a minority of the cells can influence the overall results, a possibility that was not directly considered in other studies (30, 31, 33).

Mims (48) and Gresser and Lang (49) have reviewed well the possible role of leukocytes in generalized viral infections. Although no such role has been established, it is reasonable that ingestion and destruction of virus by leukocytes may be important in host defense against influenza.

Whereas the interaction with guinea pig leukocytes varies among different influenza viruses, each of the 3 strains tested inhibited phagocytosis directly. Despite the similarity of the interaction of rat and mouse leukocytes with the different viruses, rat leukocytes resisted and mouse leukocytes were susceptible to the antiphagocytic effect of the viruses. Collectively, these results indicate that neither attachment of virus, action of viral neuraminidase, nor ingestion of virus accounts solely for the inhibition of phagocytosis. Even in a single species, the results exclude the singular responsibility of either viral neuramindiase

or ingestion in the inhibition of phagocytosis, i.e., NWS virus is neuraminidase deficient but inhibits phagocytosis by guinea pig leukocytes, and WS virus does not seem to enter guinea pig PMN but does inhibit phagocytosis. How the influenza virus impairs leukocyte function and the basis of species variation in susceptibility to these effects of the virus remain unknown. In some cases, e.g., WS virus and guinea pig PMN, a surface event must be responsible. There are other examples in which an event at the external cell surface influences profoundly the internal functions of the cell, e.g., contact inhibition of mammalian cells (50, 51), the action of colicines on bacterial cells (52), and, possibly, the action of interferon on mammalian cells (53).

Fisher and Ginsberg provided evidence that linked the inhibition of phagocytosis by guinea pig PMN to the virus-induced reduction in glycolysis (28) and suggested that the virus inhibited glycolysis by interfering with the enzyme phosphohexoisomerase (28). In the present studies, however, inhibition of glycolysis and inhibition of phagocytosis due to influenza viruses were poorly correlated among different cell types and species. Greater amounts of some viruses were needed for demonstrable inhibition of glycolysis than for phagocytosis. For example, Lee allantoic fluid demonstrably inhibited phagocytosis by guinea pig PMN, but 10-fold concentrated material was required to demonstrate reduction in glycolysis; both WS and NWS viruses exhibited similar patterns of inhibitory concentrations for phagocytosis and glycolysis with guinea pig macrophages. Such discrepancies could be due simply to differences in the sensitivity

of detection of inhibition in the 2 systems. However, all 3 viruses inhibited both glycolysis and phagocytosis by guinea pig leukocytes but only glycolysis by rat PMN. Moreover, Lee inhibited phagocytosis by guinea pig leukocytes as well as the other viruses, but was less effective in reducing glycolysis.

Although the correlation between inhibition by influenza virus of glycolysis and of phagocytosis by leukocytes is imperfect, a causal relationship can not be excluded. Different leukocytes have not been compared for the relation of phagocytic function to the level of glycolysis; rat PMN may, for example, continue to ingest bacteria at levels of glycolytic metabolism insufficient to support phagocytosis by guinea pig leukocytes.

Whatever the mechanism of the inhibition of leukocyte function by the virus, the environment of the leukocyte is crucial in the manifestation of functional impairment. After incubation with influenza virus, guinea pig leukocytes in a dilute suspension ingested pneumococci normally, but those in a concentrated suspension had reduced phagocytic activity. Similar differences have been noted in phagocytic tests with leukocytes in hyperosmolar solutions (40). The way in which the two environments influence the leukocytes differently is unknown. More serum is present in relation to both cells and bacteria in the "dilute" than in the "concentrated" system, and the additional serum may modify the virus-induced cell damage. Also, the availability of nutrients is more limited and the diffusion of wastes is less efficient in the "concentrated" suspension; such factors may impose sufficient additional stress on the cells to make significant

the virus-induced functional changes. Because exudates *in vivo* may differ widely, e.g., compare the "dilute" exudates of the peritoneum and meninges with the "concentrated" exudates that may develop in the lung, the anatomic site of virus involvement is crucial to a consideration of the role of the antiphagocytic action of influenza virus *in vivo*. The natural site of influenzal infection, the respiratory tract, is ideal for there an exudate may become very concentrated during the course of pneumonia.

According to current concepts (24), phagocytosis is a major factor in the defense of the lungs against bacteria presented as a small-particle aerosol. The present results with rats are not, then, surprising for their alveolar macrophages and exudate leukocytes resist the antiphagocytic action of influenza virus. Until viral lesions appear, therefore, rats with influenza should deal with airborne pneumococci like normal rats; they did.

Although influenza virus can inhibit phagocytosis by guinea pig leukocytes, lung defenses against airborne pneumococci were unaffected in guinea pigs with influenza during that portion of the viral infection in which the virus content of the lung was maximal, but epithelial lesions and edema were minimal. Although mice with influenza clear bacteria from the lungs poorly (22-24), impaired clearance occurs only late in the viral disease when lesions are marked but virus titers in the lungs are declining. Early, before severe lesions develop, clearance of bacteria from the lungs is normal in mice with influenza (22-24) even though mouse leukocytes

are susceptible to the antiphagocytic action of the virus. Either the virus does not significantly inhibit phagocytosis or the guinea pigs and mice have sufficient reserve defenses to overcome the deficiency in phagocytosis. The present experiments do not directly implicate either. Phagocytosis is, however, sufficiently important in lung defense that significant inhibition should have been manifested 24 hr after pneumococcal exposure by increased numbers of pneumococci in the lungs of guinea pigs with influenza; this was not observed. If phagocytosis was normal, the lack of a viral effect on leukocytes probably was due either to inadequate amounts of virus in the lungs, to inadequate juxtaposition of virus and cells, or to both.

The present negative conclusions about the role in vivo of the direct antiphagocytic action of influenza virus further support the thesis that the virus-induced epithelial lesions and edema are primarily responsible for the impaired antibacterial defense of the lungs during influenza.

Conclusions:

The interaction at physiological temperature of influenza viruses with leukocytes from mice, rats, and guinea pigs was complex. Leukocytes differed both by type and species in the way in which they reacted with influenza A and B viruses. Influenza virus attached rapidly to both PMN and macrophages from all 3 species. The outcome of the interaction depended, however, on the relative rates of two competing reactions: (1) receptor destruction by viral neuraminidase followed by elution and

(ii) ingestion by the leukocyte of the attached virus followed by intracellular destruction of the virus. Influenza A (WS strain) attached and eluted from both PMN and macrophages of mice and rats and from guinea pig PMN; WS virus was ingested and destroyed by guinea pig macrophages. Influenza B (Lee strain) was ingested and destroyed by both cell types of the 3 species.

Interaction with influenza virus reduced the phagocytosis of pneumococci by exudative PMN and macrophages and by alveolar macrophages. The extent of inhibition depended on the quantity of virus and the duration of virus-cell interaction. There were, however, differences between species; the phagocytic activity of mouse and guinea pig leukocytes was reduced by virus, but that of rat cells was not.

The effect on phagocytosis was poorly correlated with inhibition by the virus of leukocyte glycolysis and was not solely due to either viral neuraminidase (RDE), i.e., $RDE^+$ and $RDE^-$ strains caused similar changes, or penetration of the cell by the virus.

Although influenza virus can inhibit phagocytosis of pneumococci by guinea pig leukocytes, lung defenses against airborne pneumococci were unaffected in guinea pigs with influenza during that portion of the viral infection in which the virus content of the lung was maximal, but epithelial lesions and edema were minimal. These negative conclusions about the role in vivo of the direct antiphagocytic action of influenza virus further support the thesis that the virus-induced epithelial lesions and edema are primarily responsible for the impaired antibacterial defense of the lungs during influenza.

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1. Conant, J. E. and Sawyer, W. D., Transformation during mixed pneumococcal infection of mice. (Abstract) Bacteriol. Proceedings, 53 (1967).

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13. ABSTRACT

Part I. The recent demonstration by others of transformation during peritoneal infection of mice by 2 genetically distinct pneumococcal strains supports the notion that transformation may be significant in pneumococcal infection in nature. These studies confirm the occurrence of transformation during mixed infection of mice and define some conditions for its occurrence and its significance.

Mice were inoculated with DNA donor (small type III capsule, low virulence, streptomycin sensitive) and recipient (noncapsulated, low virulence, streptomycin resistant) pneumococci, and the bacteremia in the mice that died was evaluated. Transformants (large type III capsule, virulent, streptomycin resistant) were isolated from up to 80% of mice that died from mixed infection. Transformation occurred in mice that received the donor and the recipient 6 hr apart; hence, active DNA was released and competence developed during growth in vivo. Transformation was detected only with progressive infection by both strains, and then transformants were few in the blood and apparently were not responsible for the death of the animals.

In doubly-infected mice treated with streptomycin, transformation was enhanced transformants numerically dominated the bacteremia and seemed to cause the death of the mice.

Transformation was also demonstrated for the first time during infection of the respiratory tract.

| 14. KEY WORDS | LINK A | | LINK B | | LINK C | |
|---|---|---|---|---|---|---|
| | ROLE | WT | ROLE | WT | ROLE | WT |
| Pneumococcus | | | | | | |
| Pneumococcal transformation | | | | | | |
| Influenza | | | | | | |
| Phagocytosis | | | | | | |
| Leukocyte | | | | | | |
| Macrophage | | | | | | |
| Combined viral-bacterial infection | | | | | | |
| Airborne Infection | | | | | | |
| Pneumonia | | | | | | |

Abstract (continued)

Part II. Infection of the respiratory tract by influenza virus enhances susceptibility to bacterial infection, e.g., pneumococcal pneumonia. This synergism has been attributed to (i) destruction of the respiratory epithelium by the virus and (ii) the accumulation of edema in the influenza-infected lungs. In vitro studies by others with guinea pig polymorphonuclear leukocytes (PMN) suggested that a third factor may be important, namely a direct antiphagocytic action of the virus on the cells. These studies were directed at defining further the effect of influenza virus on leukocytes and determining the role of the direct antiphagocytic action of influenza virus in the heightened susceptibility to bacterial infection that accompanies influenza.

The interaction at physiological temperature of influenza viruses with leukocytes from mice, rats, and guinea pigs was complex. Leukocytes differed both by type and species in the way in which they reacted with influenza A and B viruses. Influenza virus attached rapidly to both PMN and macrophages from all 3 species. The outcome of the interaction depended, however, on the relative rates of two competing reactions: (i) receptor destruction by viral neuraminidase followed by elution and (ii) ingestion by the leukocyte of the attached virus followed by intracellular destruction of the virus. Influenza A (WS strain) attached and eluted from both PMN and macrophages of mice and rats and from guinea pig PMN; WS virus was ingested and destroyed by guinea pig macrophages. Influenza B (Lee strain) was ingested and destroyed by both cell types of the 3 species.

Interaction with influenza virus reduced the phagocytosis of pneumococci by exudative PMN and macrophages and by alveolar macrophages. The extent of inhibition depended on the quantity of virus and the duration of virus-cell interaction. There were, however, differences between species; the phagocytic activity of mouse and guinea pig leukocytes was reduced by virus, but that of rat cells was not.

The effect on phagocytosis was poorly correlated with inhibition by the virus of leukocyte glycolysis and was not solely due to either viral neuraminidase (RDE), i.e., $RDE^+$ and $RDE^-$ strains caused similar changes, or penetration of the cell by the virus.

Although influenza virus can inhibit phagocytosis of pneumococci by guinea pig leukocytes, lung defenses against airborne pneumococci were unaffected in guinea pigs with influenza during that portion of the viral infection in which the virus content of the lung was maximal, but epithelial lesions and edema were minimal. These negative conclusions about the role in vivo of the direct antiphagocytic action of influenza virus further

Abstract (continued)

support the thesis that the virus-induced epithelial lesions and edema are primarily responsible for the impaired antibacterial defense of the lungs during influenza.

# SUPPLEMENTARY

# INFORMATION

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