AD NUMBER

AD404855

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov’t. agencies and their contractors; Administrative/Operational Use; APR 1963. Other requests shall be referred to Commanding Officer, Biological Laboratories, Fort Detrick, Frederick, MD.

AUTHORITY

BDRL D/A ltr, 27 Sep 1971

THIS PAGE IS UNCLASSIFIED
NOTICE: When government or other drawings, specifications or other data are used for any purpose other than in connection with a definitely related government procurement operation, the U. S. Government thereby incurs no responsibility, nor any obligation whatsoever; and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use or sell any patented invention that may in any way be related thereto.
SYNERGISTIC ACTIVITY
BETWEEN PR8 INFLUENZA VIRUS
AND STAPHYLOCOCCUS AUREUS
IN THE GUINEA PIG

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK
The work reported here was performed under Project AX99-26-001, "Basic Research in Life Sciences" Task 02, "Basic Research Microbiology." The expenditure order was 2039.

Robert James Janssen
William Adrian Chappell
Peter John Gerone

Virus and Rickettsia Division
DIRECTOR OF BIOLOGICAL RESEARCH

Project 1A012501B02802 April 1963
This document or any portion thereof may not be reproduced without specific authorization from the Commanding Officer, Biological Laboratories, Fort Detrick, Frederick, Maryland; however, DDC is authorized to reproduce the document for U.S. Government purposes.

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

DDC AVAILABILITY NOTICE

Qualified requestors may obtain copies of this document from DDC.

Foreign announcement and dissemination of this document by DDC is limited.
ABSTRACT

The synergistic interaction of influenza virus (Type A, PR8 strain) and *Staphylococcus aureus* in guinea pigs has been studied by aerosol exposures to mixtures of the organisms. The reaction of the host was a toxic death occurring within 48 hours. Quantitative studies of this phenomenon have shown that the concentration of virus administered was critical. On the other hand, inactivated or partially inactivated staphylococci, in combination with the virus, were capable of inducing high mortality in guinea pigs. No significant differences were observed between the multiplication of either organism and the corresponding single-agent controls. Evidence suggesting a possible mechanism of toxic action is discussed.
CONTENTS

Abstract ................................................................. 3

I. INTRODUCTION .......................................................... 7

II. MATERIALS AND METHODS ............................................... 7
    A. Materials .......................................................... 7
       1. Guinea Pigs .................................................. 7
       2. Virus .......................................................... 7
       3. Bacterium ..................................................... 7
    B. Methods .......................................................... 7
       1. Exposure of Guinea Pigs to Aerosols ......................... 7
       2. Titration of Virus ........................................... 8
       3. Bacterial Counts .............................................. 8
       4. Serological Methods ........................................ 8

III. EXPERIMENTAL RESULTS .............................................. 8
    A. Response of Guinea Pigs to the Combination .................... 8
    B. Viral and Bacterial Multiplication and Formation of Lesions .... 9
    C. Varying the Viral or Bacterial Inoculum ....................... 11
    D. Varying the Interval Between Exposures ....................... 13
    E. Inactivated Inocula ........................................... 14
    F. Immunological Studies ........................................ 16

IV. DISCUSSION .......................................................... 16

V. SUMMARY ............................................................ 19

Literature Cited ....................................................... 21

FIGURES

1. Mortality in Guinea Pigs Exposed to PR8 Influenza Virus and Staphylococcus Aureus simultaneously or with PR8 Influenza Virus Alone .................................................. 10

TABLES

I. Guinea Pig Mortality After Exposure to Aerosols of Influenza Virus (PR8) and Staphylococcus Aureus ........................................ 9

II. Viral and Bacterial Content and Consolidation of Lungs From Guinea Pigs Sacrificed at 4-Hour Intervals After Exposures to PR8 Virus, Staphylococcus, or PR8 Virus Plus Staphylococcus ........ 12
III. Effects of Various Doses of Influenza Virus in Combination with Staphylococcus aureus ........................................ 13

IV. Effects of Varying the Interval Between Aerosol Exposures To PR8 Influenza Virus and Staphylococcus aureus on the Mortality in the Guinea Pigs Exposed ..................................... 14

V. Combination of Influenza Virus and Inactivated Staphylococcus aureus in Guinea Pigs .......................... 15

VI. Challenge with PR8 Influenza Virus Plus Staphylococcus in Guinea Pigs Previously Exposed to PR8 Influenza Virus or Staphylococcus .................................................. 17
I. INTRODUCTION

During the outbreak of Asian influenza in 1957, cases of influenza pneumonia were frequently complicated by concomitant infections with staphylococci.\textsuperscript{1-3} A high mortality rate among these patients suggested the possibility of a synergistic relationship between these agents. Similar results were produced experimentally in guinea pigs by exposing animals to aerosols of both influenza virus and Staphylococcus aureus. Interactions of these infectious entities are described in this report.

II. MATERIALS AND METHODS

A. MATERIALS

1. Guinea Pigs

Guinea pigs* of the Hartley strain, weighing 180 to 200 grams, were used.

2. Virus

The PR8 strain of type A influenza was used in these experiments. Prior to this study, it had been passed numerous times in mice and eggs, and eight times alternately in the allantoic cavity of eggs and in the lungs of guinea pigs.

3. Bacterium

The Staphylococcus aureus was a strain isolated from the lungs of a patient during the 1957 epidemic of Asian influenza (phage types 52, 38, and 7). Stock cultures were grown on nutrient agar, pH 6.8, and were stored at 5°C. For each experiment, colonies were picked from agar cultures and inoculated into Bacto heart infusion broth (BHIB) (pH 7.8). After incubation at 37°C for about 24 hours, broth cultures were centrifuged and the bacteria were resuspended in either virus-infected or normal allantoic fluid for aerosolization.

B. METHODS

1. Exposure of guinea pigs to aerosols

Guinea pigs were exposed to aerosols in the apparatus described by Gerone et al.* The chamber was large enough to expose eight guinea pigs.

* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.
pigs simultaneously to dynamic aerosols generated from a glass atomizer (De Vilbiss No. 40). Dow-Corning A antifoam was used in spray suspensions to minimize foaming during nebulization. Animals were exposed to aerosols for 60 minutes unless otherwise indicated.

2. Titration of Virus

Influenza virus was titrated in either 10-day or 11-day-old embryonated eggs. Tenfold dilutions of viral suspensions were made in BHIB that contained 10,000 units of penicillin and 5000 micrograms of streptomycin per milliliter. For each dilution of viral suspension, each of ten eggs was inoculated with 0.1 milliliter into the allantoic sac. After incubation of the eggs at 37°C for about 48 hours, they were refrigerated overnight and allantoic fluid from each egg was tested for the presence of hemagglutinins by the Salk method. The median egg infective dose (EID₅₀) was calculated by the method of Reed and Muench.

3. Bacterial Counts

The concentrations of S. aureus in inocula and in lungs of guinea pigs were determined by plate counts on nutrient agar. Ten per cent suspensions of lungs from infected guinea pigs were made in BHIB on a weight to volume basis. All bacterial and viral concentrations of lungs were expressed as the microbial content in one milliliter of 10 per cent tissue suspensions.

4. Serological Methods

Samples of blood were obtained by cardiac puncture. Sera were separated from cellular elements, heated at 56°C for 30 minutes, and titrated by the hemagglutination-inhibition technique of Hirst. The antibody was assayed in the presence of four hemagglutinating doses of PR8 viral antigen.

In the complement-fixation tests, 0.2 ml of each reagent was used to make a total volume of one milliliter. Complete fixation was used as the endpoint after incubation overnight at 4°C.

III. EXPERIMENTAL RESULTS

A. RESPONSE OF GUINEA PIGS TO THE COMBINATION

The synergistic action of PR8 influenza virus and Staphylococcus aureus in guinea pigs exposed for 60 minutes to aerosols of these organisms is shown in Table I. These data are a composite result of several experiments. The per cent mortality was increased and the time-to-death was decreased in the animals exposed to the combined inocula as compared with the animals exposed to influenza virus alone. Figure 1 more clearly illustrates the synergistic phenomenon expressed as a reduction of the time-to-death of animals exposed to the virus-bacteria combination as compared with those
TABLE I. GUINEA PIG MORTALITY AFTER EXPOSURE TO AEROSOLS OF INFLUENZA VIRUS (PR8) AND STAPHYLOCOCCUS AUREUS

<table>
<thead>
<tr>
<th>GROUP</th>
<th>AEROSOL INOCULUM a/</th>
<th>NUMBER EXPOSED</th>
<th>DEATHS ON DAYS FOLLOWING EXPOSURE</th>
<th>PER CENT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>PR8 + S. aureus</td>
<td>75</td>
<td>23</td>
<td>39</td>
</tr>
<tr>
<td>B</td>
<td>PR8</td>
<td>85</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>C</td>
<td>S. aureus</td>
<td>21</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

a. Spray suspension for group A contained about $10^{7.5}$ EID₅₀ of PR8 per ml and approximately $2 \times 10^9$ S. aureus per ml. Equivalent viral and bacterial concentrations were used for groups B and C. Exposure time: 60 minutes.

Exposed to the virus alone. Deaths recorded at four-hour intervals were first observed in guinea pigs exposed to both organisms during the interval of 16 to 20 hours after exposure. The earliest deaths due to influenza virus occurred during the 24- to 28-hour interval. Prior to death, guinea pigs in the influenza control group and those that received the combination exhibited pronounced respiratory difficulties. Although elevations in body temperature were uniformly absent, subnormal temperatures were recorded immediately preceding death. Necropsies of guinea pigs revealed massive lung consolidation in animals exposed to both agents as well as in those exposed only to the virus. No fatalities occurred in the animals receiving staphylococci alone and lesions were not observed in the lungs of animals sacrificed at intervals corresponding to those in the other two groups.

B. VIRAL AND BACTERIAL MULTIPLICATION AND FORMATION OF LESIONS

Experiments were designed to determine whether the rapid deaths that occurred in animals exposed to the combination of influenza virus and staphylococcus were due to, or resulted from: (a) a difference in rate or total multiplication of one or both of the microorganisms, or (b) a difference in the severity of the lesions formed in principals as compared with the control animals. Three groups of eight guinea pigs each were exposed to influenza virus, or staphylococcus, or influenza virus plus staphylococcus, for 60 minutes in the aerosol apparatus. Immediately after exposure and at four-hour intervals for a period of 28 hours, one animal from each group was selected at random and sacrificed. A blood sample from each guinea pig was plated on blood agar to test for bacteremia. The lungs were removed aseptically in toto and were washed once in sterile saline to remove excess blood and examined for gross lesions. The lungs were ground aseptically in TenBroeck tissue grinders and 10 per cent
Figure 1. Mortality in Guinea Pigs Exposed to PR8 Influenza Virus and Staphylococcus aureus Simultaneously or with PR8 Influenza Virus Alone. No deaths occurred following exposure to Staphylococcus aureus alone.
suspensions were prepared in BHIB. One aliquot of the suspension was used for bacterial counts and the other, treated with penicillin and streptomycin, was inoculated into 10-day-old embryonated eggs for titration of virus. The results obtained from this experiment are presented in Table II. A greater degree or rate of multiplication of influenza virus did not appear to be responsible for the synergistic phenomenon, because virus multiplication was greater in the animals receiving virus alone than in those receiving the combination. The bacteria did not multiply significantly in the control animals, as can be seen from the decreasing counts over the 28-hour period. A more rapid reduction of viable cocci occurred in the lungs of the animals exposed to the virus-bacteria combination than in controls. A tentative explanation for this rapid clearance will be discussed in a later section of this paper. Lesions were much more pronounced in the animals that received the combination than in the control animals. Bacteria were never isolated from blood samples.

C. VARYING THE VIRAL OR BACTERIAL INOCULUM

Preliminary studies indicated that aerosol exposure for periods of 45 to 60 minutes to undiluted allantoic fluid containing influenza virus was necessary to cause deaths in guinea pigs. Experiments were performed to determine whether such a long exposure to aerosols containing high-titered virus was necessary for the virus-staphylococcus combination to induce mortality. Preliminary experiments indicated that synergistic responses were not elicited when guinea pigs were exposed to aerosols of influenza virus for 15 or 30 minutes and subsequently exposed to the bacteria for 60 minutes. Since synergistic reactions did not occur with decreased exposure time, an attempt was made to determine the effect of varying the viral inoculum without decreasing the exposure time. Dilutions (1:2 through 1:10) of influenza virus were prepared in sterile BHIB or in a 24-hour BHIB culture of staphylococcus. Guinea pigs were exposed for 50 minutes to the various suspensions. The results obtained from this experiment are presented in Table III. More pronounced synergistic effects were observed when high concentrations of influenza virus were present in the spray inocula but it was possible for the combination to kill animals that would not die from exposure to equivalent concentrations of virus or bacteria alone. The concentration of influenza virus required to kill 100 per cent of animals when used in combination with staphylococci was apparently quite critical. This could not be accomplished with a two-fold dilution that represented a rather small decrease in the number of active virus particles in the suspension.

In experiments in which the staphylococcal concentration of the suspension was varied, results were obtained that were difficult to interpret. High mortality that was due to influenza virus alone tended to obscure the results. In general the concentration of staphylococci was not as critical as the viral concentration and comparable results were obtained when the suspensions contained $1.0 \times 10^6$ to $1.0 \times 10^7$ viable bacteria per milliliter. Synergism was not demonstrated when the exposure time to the bacteria was decreased to less than 60 minutes or when a
<table>
<thead>
<tr>
<th>TIME OF SACRIFICE</th>
<th>PR8 titer ((\log_{10}))</th>
<th>S. aureus viable lung counts</th>
<th>PR8 + S. aureus viable lung counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>spray suspension</td>
<td>9.3</td>
<td>5.3 x 10^8</td>
<td>9.4</td>
</tr>
<tr>
<td>0 hr</td>
<td>4.9</td>
<td>-</td>
<td>2.6 x 10^6</td>
</tr>
<tr>
<td>4 hr</td>
<td>4.8</td>
<td>-</td>
<td>6.2 x 10^6</td>
</tr>
<tr>
<td>8 hr</td>
<td>5.6</td>
<td>-</td>
<td>5.4 x 10^6</td>
</tr>
<tr>
<td>12 hr</td>
<td>6.9</td>
<td>-</td>
<td>1.3 x 10^6</td>
</tr>
<tr>
<td>16 hr</td>
<td>7.1</td>
<td>1+</td>
<td>2.5 x 10^5</td>
</tr>
<tr>
<td>20 hr</td>
<td>7.7</td>
<td>±</td>
<td>4.3 x 10^5</td>
</tr>
<tr>
<td>24 hr</td>
<td>8.0</td>
<td>1+</td>
<td>2.0 x 10^5</td>
</tr>
<tr>
<td>28 hr</td>
<td>7.4</td>
<td>±</td>
<td>1.0 x 10^5</td>
</tr>
</tbody>
</table>

a. Sixty-minute aerosol exposures; virus titers in EID\(_{50}\) per ml \(\log_{10}\) of 10 per cent guinea pig lung; bacterial concentration in viable cell counts per ml of 10 per cent guinea pig lung.

b. Indicates no consolidation, ± less than 25 per cent, 1+ approximately 25 per cent, 2+ approximately 50 per cent, 3+ approximately 75 per cent.
TABLE III. EFFECTS OF VARIOUS DOSES OF INFLUENZA VIRUS IN COMBINATION WITH STAPHYLOCOCCUS AUREUS

<table>
<thead>
<tr>
<th>EXPOSURE SUSPENSION</th>
<th>DILUTIONS OF PR8</th>
<th>NUMBER OF GUINEA PIGS</th>
<th>DEATHS BY DAYS</th>
<th>MORTALITY RATIO dead/exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR8</td>
<td>1:2</td>
<td>8</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>PR8</td>
<td>1:4</td>
<td>12</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>PR8</td>
<td>1:6</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PR8</td>
<td>1:8</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. aureus</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PR8 + S. aureus</td>
<td>1:2</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>PR8 + S. aureus</td>
<td>1:4</td>
<td>8</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>PR8 + S. aureus</td>
<td>1:6</td>
<td>4</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>PR8 + S. aureus</td>
<td>1:8</td>
<td>4</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>PR8 + S. aureus</td>
<td>1:10</td>
<td>4</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

a. Sixty-minute aerosol exposures.
b. S. aureus concentration approximately $5 \times 10^8$ cells per ml.

Sintered glass filtrate of the staphylococcal culture was used in the aerosol exposures. The latter observation suggested that the synergistic response was not caused by a product in the spent medium in which the staphylococci were cultivated.

D. VARYING THE INTERVAL BETWEEN EXPOSURES

The data presented above have been concerned with exposure of animals to the mixture of influenza virus and Staphylococcus aureus. Experiments were devised to determine whether simultaneous exposure was necessary to exhibit synergism. Table IV presents the results obtained when exposure to the bacterial suspension was made immediately after (0-hour) and at 6, 24, and 48 hours after the initial exposures to influenza virus.
TABLE IV. EFFECTS OF VARYING THE INTERVAL BETWEEN AEROSOL EXPOSURES TO PR8 INFLUENZA VIRUS AND STAPHYLOCOCCUS AUREUS ON THE MORTALITY IN THE GUINEA PIGS EXPOSED

<table>
<thead>
<tr>
<th>FIRST INOCULUM</th>
<th>SECOND INOCULUM</th>
<th>HOURS BETWEEN EXPOSURES</th>
<th>MORTALITY RATIO dead/exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR8</td>
<td>S. aureus</td>
<td>0</td>
<td>4/4</td>
</tr>
<tr>
<td>PR8</td>
<td>S. aureus</td>
<td>6</td>
<td>4/4</td>
</tr>
<tr>
<td>PR8</td>
<td>S. aureus</td>
<td>24</td>
<td>4/4</td>
</tr>
<tr>
<td>PR8</td>
<td>S. aureus</td>
<td>48</td>
<td>0/4</td>
</tr>
</tbody>
</table>

a. Sixty-minute aerosol exposure.

Exposure to staphylococci elicited a synergistic response in the guinea pigs that were exposed to the virus as much as 24 hours previously. Synergism could not be demonstrated when exposure to staphylococci preceded exposure to influenza virus.

E. INACTIVATED INOCULA

Because the most consistent synergistic effects were observed with large inocula of bacteria, experiments were carried out to test the possibility that the total cell concentration was important rather than the viable cell count. Results from these experiments are presented in Table V. Two methods of inactivation were used. Partial inactivation of the staphylococci was achieved by using a combination of sonic vibration (Six hours at maximum frequency in a Raytheon 9+K.8. sonic oscillator) and ultraviolet irradiation (30 minutes at a distance of two inches from a UV source). A second, more effective method of inactivation was heating the suspension of staphylococci for 30 minutes at 60°C. Suspensions that were treated to make the cells partially or totally nonviable were still synergistic in combination with the virus. These results suggest that it was the total bacterial concentration and not the viable cells that was necessary for synergism. One of the controls in this experiment consisted of exposing animals to a bacterial concentration comparable to that of the partially inactivated staphylococcal inoculum (3.0 x 10^7). Mortalities obtained with this preparation mixed with...
TABLE V. COMBINATION OF INFLUENZA VIRUS AND INACTIVATED STAPHYLOCOCCUS AUREUS IN GUINEA PIGS a/  

<table>
<thead>
<tr>
<th>AEROSOL INOCULUM b/</th>
<th>INACTIVATION PROCEDURE</th>
<th>S. AUREUS CONCENTRATION AFTER INACTIVATION</th>
<th>MORTALITY RATIO Dead/exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR8</td>
<td>-</td>
<td>-</td>
<td>2/16</td>
</tr>
<tr>
<td>S. aureus</td>
<td>-</td>
<td>1.7 x 10^9</td>
<td>0/4</td>
</tr>
<tr>
<td>S. aureus</td>
<td>sonic vibrations UV irradiation</td>
<td>7.6 x 10^3</td>
<td>0/4</td>
</tr>
<tr>
<td>PR8 + S. aureus</td>
<td>-</td>
<td>1.7 x 10^9</td>
<td>4/4</td>
</tr>
<tr>
<td>PR8 + S. aureus</td>
<td>sonic vibrations UV irradiation</td>
<td>7.6 x 10^3</td>
<td>4/4</td>
</tr>
<tr>
<td>PR8 + S. aureus</td>
<td>-</td>
<td>3.0 x 10^3</td>
<td>1/4</td>
</tr>
<tr>
<td>PR8 + S. aureus</td>
<td>heat, 60°C for 30 min</td>
<td>0</td>
<td>6/8</td>
</tr>
<tr>
<td>PR8</td>
<td>heat, 60°C for 30 min</td>
<td>-</td>
<td>0/4</td>
</tr>
<tr>
<td>PR8 + S. aureus</td>
<td>heat, 60°C for 30 min</td>
<td>4.2 x 10^8</td>
<td>0/4</td>
</tr>
</tbody>
</table>

a. Also inactivated PR8 virus alone and in combination with live S. aureus.
b. Sixty-minute aerosol exposures.
c. PR8 inactivated, not S. aureus.

The influenza virus were similar to those of the influenza virus controls and strengthened the hypothesis that total and not viable cell concentration was the important factor in this phenomenon. Data reported in the earlier section on effects of diluting the staphylococci were somewhat contradictory to these results; however, excessive mortalities in all these cases could be accounted for on the basis of influenza virus alone and were not necessarily indicative of effects from the combined infection. Death was not observed when either heat- or ultraviolet-inactivated influenza virus was used.
In the section on multiplication of agents (Table II), it was noted that bacteria were cleared more rapidly from the lungs of animals exposed to the combination of influenza virus and staphylococci than from control animals that were exposed to the bacteria alone. The more rapid elimination could be explained on the basis of the smaller concentration of the bacteria in the lungs of animals that received the combination, as evidenced by the titers at zero-hour in this group. However, a question remained as to why the viable bacterial concentration should be lower in the lungs of animals exposed to the combination (zero-hour titer of $4.3 \times 10^6$ per ml) than in the bacterial controls (zero-hour titer of $2.6 \times 10^6$ per ml) when the aerosol inocula contained similar concentrations of staphylococci. Because the inoculum was prepared by mixing the viral and bacterial cultures, two possibilities for the decrease in recoverable bacteria were considered. Either an antibacterial effect of the influenza virus manifested itself or the cocci were inactivated by residual penicillin and/or streptomycin used in the preparation of the influenza virus pool. It was determined in a separate experiment that residual antibiotic used in the preparation of the virus pool and present in the mixture of virus and bacteria was sufficient to account for the lower bacterial counts.

F. IMMUNOLOGICAL STUDIES

Guinea pigs that survived exposure for varying periods of time to aerosols of influenza virus developed complement-fixing antibody titers of 64 to 1280 and hemagglutination-inhibiting antibody titers of 1280 to 2560. These titers were observed in all sera collected as early as 14 days and as late as 56 days following exposure. No antibodies were detectable in the sera of non-exposed animals or in the sera of animals exposed to normal chorio-allantoic fluids. In these particular experiments, serological studies could not be performed with sera from animals exposed to the viral-bacterial combinations because none of these animals survived long enough to develop demonstrable antibody.

Guinea pigs that survived exposures to various concentrations of influenza virus or staphylococci were challenged to determine whether previous exposure to either agent influenced their resistance to the combination. Challenges were performed 14 days after initial exposures. The following conclusions may be drawn from the data presented in Table VI. Previous exposures to the staphylococci alone induced no protection against combined infection. Survivors from exposures of 60 minutes or less to diluted influenza virus preparations showed little resistance to the combined infections, but guinea pigs that survived a 60-minute exposure to undiluted influenza virus were completely protected against the combined challenge.

IV. DISCUSSION

Synergism, in the studies described in this paper, has been defined as an increased susceptibility of guinea pigs exposed to aerosols containing a combination of influenza virus and staphylococci as compared with the susceptibility of these animals exposed to aerosols of either agent alone.
TABLE VI. CHALLENGE WITH PR8 INFLUENZA VIRUS PLUS STAPHYLOCOCCUS a/ IN GUINEA PIGS PREVIOUSLY EXPOSED TO PR8 INFLUENZA VIRUS OR STAPHYLOCOCCUS

<table>
<thead>
<tr>
<th>INITIAL EXPOSURE</th>
<th>CHALLENGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>aerosol inoculum</td>
<td>dilution sprayed</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Undiluted</td>
</tr>
<tr>
<td>PR8</td>
<td>Undiluted</td>
</tr>
<tr>
<td>PR8</td>
<td>Undiluted</td>
</tr>
<tr>
<td>PR8</td>
<td>Undiluted</td>
</tr>
<tr>
<td>PR8</td>
<td>1:2 - 1:10 b/</td>
</tr>
</tbody>
</table>

a. Challenge exposure: 14 days after initial exposure, 60 minutes exposure.
b. Exposed to 1:2, 1:4, 1:8, or 1:10 dilution - mortality similar within each group.

The mortality patterns observed in the animals exposed to the virus-bacteria combination or to the virus alone were suggestive of a toxic reaction rather than of an infectious process. The evidence suggesting this included the following: first, to cause death by both organisms administered simultaneously or by the virus alone, it was necessary to aerosolize high concentrations of influenza virus for a prolonged exposure (60 minutes). Any alteration of this procedure (i.e., either using a 1:2 dilution of stock virus or diminishing the exposure time to 30 or 45 minutes) decreased significantly the number of deaths observed. Second, mortalities occurred very soon after exposure, usually within 24 hours after exposure to the combination and 24 to 72 hours after exposure to virus. Although virus multiplication reached its peak in the lungs of guinea pigs 24 hours after exposure, these early death patterns were not analogous to the patterns following exposure of mice to aerosols of influenza virus. As shown by Gerone et al., mice exposed to aerosols of influenza virus died later and deaths occurred over a longer period of time. Moreover, the early deaths in guinea pigs were very similar to those seen in mice inoculated by routes other than the respiratory route (e.g., intracerebral, intraperitoneal, and intravenous) with high concentrations of influenza virus; the deaths of these animals were due to the toxic action of the virus. Third, the massive, almost complete consolidation of the lungs of guinea pigs that succumbed to the virus and bacterial combination or virus alone was similar to lesions observed following intranasal inoculation of mice with large doses of influenza virus. However, it is not necessarily valid to compare
influenza virus infections in mice with those in guinea pigs because the inherent susceptibility of the mouse appears to be much greater than that of the guinea pig and therefore the response may differ considerably.

The fact that exposure to the virus must be carried out before, or simultaneously with, the bacterial exposure suggested several interesting possible mechanisms for the synergistic reaction. Harford et al. and Gerone et al. indicated that the time of appearance of lung lesions, resulting from the influenza infection, was important in decreasing the resistance of mice to subsequent challenge with pneumococci. Whether the lung damage caused by the influenza virus in guinea pigs decreased the host's natural resistance by acting as a "portal of entry" for the staphylococcus or by some other means is presently unknown. However, it was established that killed bacteria were almost as synergistic as viable organisms, and this observation suggested that possibly a heat-stable endotoxin-like material from the bacterial cell was the important factor. Because staphylococci alone were not lethal for the guinea pig, the role of the virus in reducing the resistance of the guinea pig is again suggested. One might speculate that perhaps the virus induces lesions in the lungs of guinea pigs that permit the toxic bacterial substances to manifest their lethal effect in the animal. Confirmation of this hypothesis is not available.

Because deaths occurred within a matter of hours after exposure, the probability that staphylococci interfered with antibody production by decreasing host resistance was considered remote. It is interesting to note that resistance to the combined inoculum was observed in survivors exposed to large concentrations of influenza virus, whereas no such resistance was observed in the staphylococcus controls that were challenged with the virus-bacterium combination by the respiratory route. This fact, again, points to the important role played by the virus in this synergistic phenomenon.

Preliminary aerosol studies were carried out in mice, using as inocula the same PR8 influenza virus and Staphylococcus aureus described above. In these studies, no synergistic action was observed. In experiments with the embryonated chicken egg as a host, definite synergistic action in terms of enhanced mortality, was observed with these same two microorganisms and later with different strains of both organisms. One major difference observed in tests with eggs was the fact that synergism was demonstrated even when extremely small quantities of both microorganisms were used. The embryonated egg is apparently much more sensitive to the mixture of microorganisms than the guinea pig.
LITERATURE CITED


