Synergistic Effect in Viral-Bacterial Infection: Combined Infection of the Murine Respiratory Tract with Sendai Virus and Pasteurella pneumotropica

GEORGE J. JAKAB1 AND ELLIOT C. DICK

Departments of Medical Microbiology and Preventive Medicine, University of Wisconsin Medical School,
Madison, Wisconsin 53706

Received for publication 2 July 1973

Synergism was demonstrated between Sendai virus and Pasteurella pneumotropica in the respiratory tract of mice showing no evidence of previous infection with either agent. Mice aerosol challenged with P. pneumotropica invariably eliminated the viable organism from their lungs within 72 h. In contrast, intrapulmonary killing was delayed in animals previously infected with Sendai virus. Maximum synergism was observed when virus infection preceded bacterial challenge by 6 days. At this time, a mortality rate of 37% was observed as compared with 0, 10, 20, and 10%, respectively, in those animals in which the virus infection preceded bacterial challenge by 1, 3, 9, and 12 days. Previous immunization with Sendai virus completely prevented virus infection and thus the synergistic effect. Synergism with endogenous flora was also noted. Six days after virus infection an endogenous Pasteurella sp. began to proliferate in the bronchopulmonary tissues. Up to 10^4 colony-forming units per lung were recovered but no animals died of the endogenous Pasteurella infection.

Virus infections often predispose to bacterial disease in the lung. In combination these agents will increase host symptoms and mortality compared with those found when either agent alone is acting on the host (29). Previous experimental studies on viral-bacterial interactions in the respiratory tract have focused primarily on infection with viruses and bacteria of human origin (6, 8, 9, 13, 23, 27, 36); little attention has been given to combined experimental infection of animals with their natural pathogens.

Sendai virus and Pasteurella pneumotropica are natural infectious agents of mice (7, 20, 24, 31, 32). Both agents are frequently found in mouse colonies, but there is no reported clinical disease in naturally infected mice (11, 25, 31).

Under experimental conditions, Sendai virus-infected mice develop pathologic pulmonary changes of bronchial epithelial cell desquamation, interstitial pneumonitis, and consolidation (1, 22, 34) similar to those caused by influenza virus infection in mice (18, 30, 37). Because the pathologic changes in the lungs of mice closely resemble the lesions in influenza virus, its natural affinity for mice, and its apparent lack of pathogenicity for man, Sendai virus infection of mice makes an ideal model for the study of acute respiratory virus infections (38).

P. pneumotropica is a potential pulmonary pathogen in mice frequently associated with outbreaks of pneumonia in mouse colonies (3, 4, 19, 24, 25). Experimental studies have demonstrated that the pathogenicity of the Pasteurella organism is governed by the functional state of the pulmonary antibacterial defenses (10).

(This investigation is based on a thesis submitted by G. J. Jakab in partial fulfillment of the Ph.D. degree in Medical Microbiology at the University of Wisconsin. Portions of this investigation were presented at the 1970 Annual Meeting of the American Society for Microbiology in Boston.)

MATERIALS AND METHODS

Animals. White male Swiss mice (Ha/ICR strain) weighing 18 to 20 g and showing no serologic evidence

1 Present address: Pulmonary unit, University of Vermont College of Medicine, Department of Medicine, Burlington, Vermont 05401. Reprint requests to Dr. Jakab.
of Sendai virus infection were used. These animals did not harbor *P. pneumotropica* in their respiratory tract. Instead they were naturally infected with a *Pasteurella* sp. which differed biochemically from *P. pneumotropica* (Table 1).

**Virus.** Parainfluenza 1 (Sendai) virus obtained from the American Type Culture Collection was harvested in the allantoic fluid of 13-day-old chicken embryos after incubation at 37°C for 2 days, was titered in rhesus monkey kidney tissue cultures, and stored at −70°C in small portions.

**Bacterium.** *P. pneumotropica* was isolated from the nasopharynx of a mouse from a colony other than the one used in these studies. For aerosol infection the organism was grown in 100 ml of brain heart infusion (BHI) broth for 18 h at 37°C, was centrifuged, and resuspended in 10 ml of BHI broth.

**Animal exposure.** Airborne infection of the murine lung with either Sendai virus or *P. pneumotropica*, or both, was achieved in a previously described aerosol chamber (2). Virus doses inhaled by the animals were calculated by methods previously described (2, 15). Each animal inhaled approximately 10⁶ mean tissue culture infective doses (TCID₅₀) of Sendai virus, a dose which causes a self-limiting infection. The bacterial dose administered to each animal was the geometric mean of the actual number of organisms recovered from 3 to 6 mice sacrificed immediately after 30 min of aerosol exposure to *P. pneumotropica*.

**Bacterial enumeration and virus titration.** Lungs from sacrificed or expired mice were aseptically removed, observed for gross pathological changes, and homogenized in 4 ml of iced citrate-phosphate buffered medium 199 solution (CP/199), pH 7.0. After centrifugation at 500 × g for 5 min, 1 ml of the homogenate was diluted appropriately and cultured quantitatively on BHI sheep blood agar. In instances where lungs of the same animals were assayed for both virus and bacterial content, 0.1 ml of 10-fold dilutions in CP/199 of the homogenate supernatant fluid was inoculated into each of 2 MKTC tubes. TCID₅₀ end points were calculated by the Karber method (28).

**Experimental design.** Each individual experiment consisted of four groups of animals. The groups and the organism(s) they were infected with and the assays performed on them are depicted in Table 2. Additional groups used are appropriately noted.

All mice that died were necropsied within 1 h of death. As mice in group 3 (Table 2) were sacrificed daily the number of *P. pneumotropica* recovered from animals that died in this group was included with those recovered from the sacrificed animals in calcul-

<table>
<thead>
<tr>
<th>Group</th>
<th>Sendai virus</th>
<th><em>P. pneumotropica</em></th>
<th>Observed or assayed for</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>−</td>
<td>Mortality</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>+</td>
<td>Quantitative bacterial counts</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>Quantitative bacterial counts</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>Mortality</td>
</tr>
</tbody>
</table>

* +, Infected.
* −, Not infected.

**RESULTS**

Preliminary experiments had demonstrated that normal mice aerosol challenged with *P. pneumotropica* eliminated over 99% of the viable organisms from the lung parenchyma within 24 h. Intrapulmonary killing continued until, 72 h later, all the lungs were consistently bacteriologically sterile. Using these data, synergism, in future combined infection experiments, was defined as failure to eliminate viable *P. pneumotropica* from the lungs within 72 h.

**Intrapulmonary killing of *P. pneumotropica* 6 days after Sendai virus infection.** Experiments by others have demonstrated that failure of virus-infected lungs to eliminate viable bacteria is associated in time with maximum pathologic changes (9, 16, 17, 36) which, in the case of Sendai virus, developed approximately 6 days after infection. Accordingly, in two separate experiments, mice were aerosol challenged with *P. pneumotropica* 6 days after infection with Sendai virus (Fig. 1). Marked synergism was observed in virus-infected mice as indicated by the stasis of the bacterial population at zero time levels for 3 days after bacterial challenge and by greatly delayed elimination of viable bacteria as compared with bacterial elimination from the lungs of non-virus-infected mice.

**Intrapulmonary killing of *P. pneumotropica* 1, 3, 6, 9, and 12 days after Sendai virus infection**...
infection. To determine the optimal time for synergism, mice were aerosol challenged with *P. pneumotropica* at 1-, 3-, 6-, 9-, and 12-day intervals after Sendai virus infection.

Synergism was observed in all groups as none of the mice tested eliminated the viable bacteria from their lungs within 72 h in contrast to those that were administered *P. pneumotropica* alone (Fig. 2). Optimal synergism was observed when the infectious agents were given at a 6-day interval. Two days after the 6-day group was challenged with *P. pneumotropica* the pulmonary bacterial content was still at inoculum levels which was 1000-fold greater than in the other groups. Maximal deaths also occurred in the 6-day group (Table 3).

**Effect of viral "immunization" on synergism.** To determine the effect of previous viral infection on subsequent viral bacterial interactions in the lung, mice were initially

**Fig. 1.** Intrapulmonary killing of *P. pneumotropica* in mice infected with Sendai virus 6 days prior to bacterial challenge. Each point represents the geometric mean of bacterial counts in the lungs of three mice.

**Fig. 2.** Intrapulmonary killing of *P. pneumotropica* in mice challenged with the bacterium at 1, 3, 6, 9, and 12 days after Sendai virus infection. Each point represents the geometric mean of bacterial counts in the lungs of three mice.
infected with $4 \times 10^2$ TCID$_{50}$ of Sendai virus. Serum hemagglutination inhibition antibody levels against Sendai virus 13 days after infection ranged from 1:8 to 1:128 with the geometric mean titer for the group being 1:32. On day 14 the previously infected mice were reinfected with $10^4$ TCID$_{50}$ of virus followed 6 days later by aerosol challenge with \textit{P. pneumotropica}. Previous infection with Sendai virus afforded complete protection against subsequent combined challenge as the virus-immunized animals eliminated \textit{P. pneumotropica} just as rapidly as the control group receiving the bacterium alone (Fig. 3).

**Effect of \textit{P. pneumotropica} infection on pulmonary titers of \textit{Sendai} virus.** Having established that Sendai virus infection delays the subsequent elimination of the superinfecting \textit{P. pneumotropica}, an experiment was performed to determine the effect of concurrent \textit{P. pneumotropica} infection on Sendai virus elimination. Mice were initially infected with $10^4$ TCID$_{50}$ of Sendai virus and divided into two groups. Animals from one group received no further treatment and were sacrificed daily for 14 days to obtain pulmonary virus titers. The other group of mice were challenged with \textit{P. pneumotropica} 6 days after virus infection. Immediately after bacterial infection and at daily intervals thereafter both pulmonary virus titers and \textit{P. pneumotropica} counts were determined on the sacrificed mice.

Maximal virus titers of $10^7$ TCID$_{50}$ per lung, recovered from day 3 through day 5 after Sendai virus infection, and were rapidly diminished by day 9, after which no infectious virus could be recovered. Superinfection with \textit{P. pneumotropica} had no effect on viral elimination (Fig. 4).

**Emergence of an endogenous \textit{Pasteurella} sp. in the lungs of \textit{Sendai} virus-infected animals.** To detect possible bacterial contamination, lung homogenates of Sendai virus-infected animals were also plated on BHI sheep blood agar. Through day 5, lungs of virus-infected animals remained bacteriologically sterile. By day 6, however, the lungs of one of the three sacrificed mice contained alpha hemolytic streptococci, diptheroids, and the \textit{Pasteurella} sp. indigenous to this particular mouse colony. On day 7 a greater number of these organisms were recovered from two of the three sacrificed mice and by day 8 pure cultures of the \textit{Pasteurella} sp. were found in two mice, whereas the third contained the above assortment. After day 8 these endogenous organisms declined until by day 11 the lungs were again bacteriologically sterile (Fig. 4).

**Table 3. Total mortality of mice infected with Sendai virus and \textit{P. pneumotropica} in combination or singly.**

<table>
<thead>
<tr>
<th>Infected with</th>
<th>No. mice died/no. mice infected</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sendai virus only</td>
<td>1/70</td>
<td>1.4</td>
</tr>
<tr>
<td>\textit{P. pneumotropica} only</td>
<td>0/65</td>
<td>0</td>
</tr>
<tr>
<td>Sendai virus + \textit{P. pneumotropica} (virus immunized)</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>\textit{P. pneumotropica} 1 day after Sendai virus</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>\textit{P. pneumotropica} 3 days after Sendai virus</td>
<td>1/10</td>
<td>10</td>
</tr>
<tr>
<td>\textit{P. pneumotropica} 6 days after Sendai virus</td>
<td>26/70</td>
<td>37</td>
</tr>
<tr>
<td>\textit{P. pneumotropica} 9 days after Sendai virus</td>
<td>2/10</td>
<td>20</td>
</tr>
<tr>
<td>\textit{P. pneumotropica} 12 days after Sendai virus</td>
<td>1/10</td>
<td>10</td>
</tr>
</tbody>
</table>

**Pulmonary changes.** \textit{P. pneumotropica} infection alone produced no overt histopathology. In contrast, Sendai virus infection at 6 days was marked by increasing bronchial epithelial cell desquamation, interstitial pneumonitis, atelectasis, and scattered areas of consolidation. Sendai-immunized mice, subsequently reinfected with Sendai virus, showed no gross lung pathology.

Twenty-four hours after superimposing \textit{P. pneumotropica} on the 6-day-old Sendai virus infection, peribronchial regions were observed to be intensely infiltrated by polymorphonuclear cells. Within 48 h some of the bronchi were completely occluded with a purulent exudate and the peribronchial inflammatory infiltrate was spreading to adjacent alveoli. Areas of consolidation in the lung parenchyma were also enlarging. By day 3 the congestion was still spreading. The histologic picture in lungs of mice succumbing to the infection at this time was that of confluent consolidation accompanied by severe hemorrhage in over 50% of the lung.

**DISCUSSION**

In pulmonary bacterial infections the initial host-parasite encounter is a critical event in determining the outcome of the disease. If the hosts' defense mechanisms remove or kill the organism the potential infection is averted. If the viable organism is not eliminated the bacteria may proliferate causing bacterial pneumonitis. Pulmonary virus infections are known to predispose to bacterial infections in the lung.
In this study we have investigated viral-bacterial interaction in the respiratory tract of mice with their natural infectious agents.

The data presented in this investigation clearly demonstrate that sequential infection of the respiratory tract of mice with Sendai virus and \textit{P. pneumotropica} decreased the ability of the lungs to eliminate viable bacteria. Furthermore, this synergistic effect was demonstrated by an enhanced mortality as compared with that of virus or bacteria alone. Mice succumbing to the combined infection consistently had evidence of pneumonia.

Virus infection impairs the elimination of viable bacteria, whereas concurrent bacterial infection has no effect on elimination of infectious virus from the lungs. These results indicate that concomitant bacterial infection does
not enhance the pathogenicity of the viral process. On the other hand, the viral process may enhance the bacterial infection by selecting pathogenic forms of the organism (5). However, this appears unlikely since impairment of pulmonary bactericidal activity by noninfectious methods, such as nephrectomy, also allows the immediate proliferation of *P. pneumotropica* after aerosol challenge (10).

A positive correlation was established between gross and microscopic pathologic changes and an optimal synergistic effect. Previous immunization with Sendai virus prevented the induction of these gross pathologic changes upon subsequent infection with the virus and resulted in a failure to reproduce the synergistic effect during a subsequent dual infection. These data indicate that an active pulmonary infection with the virus is necessary for the observed synergism.

Because of the correlation between maximal viral-induced pathologic changes and optimal suppression of pulmonary bactericidal activity, it has been commonly held that failure of viral-infected lungs to clear inactivated bacteria was due to either impaired physical transport caused by bronchial epithelial desquamation, a decreased antibacterial response from the host, or an environment which was more favorable for bacterial multiplication, or both.

The findings of Green and Kass (14) concerning the disproportionate decrease in radiotracer and viable staphylococci from the lungs of mice exposed to 32P-labeled *Staphylococcus aureus* focused on the importance of intrapulmonary bactericidal mechanisms in contrast to transport as the main defense mechanisms in the lungs against bacterial infections. Virus infection does not impair the transport of radiolabeled bacteria from virus-infected lungs despite histologic evidence of extensive destruction of bronchial-ciliated epithelium, indicating that bacterial multiplication associated with virus infection of lungs is related to defects in pulmonary bactericidal mechanisms (22). In situ bactericidal mechanisms of the lung have been attributed to the pulmonary macrophage (14) which kills, inactivates, or limits the growth of infectious organisms (12). In vitro studies suggest that virus infections suppress the bactericidal functions of the phagocytes (35), whereas in vivo studies also suggest a direct anti-phagocytic action of virus on the alveolar phagocyte (G. J. Jakab and G. M. Green, 1973, submitted for publication).

The spontaneous appearance and multiplication of endogenous *Pasteurella* in the pulmonary tissue of mice previously infected with Sendai virus verifies the synergism in viral-bacterial interactions seen with exogenously imposed *P. pneumotropica*. Similar results have been reported in the past when host resistance mechanisms were lowered by previous virus infection (27, 33, 36) or by placing mice in "cold wet air" (26). Although in most instances the gram-negative organism recovered from the lungs of mice remained unidentified, descriptions such as "gram-negative cocccobacilli" resembling *Haemophilus influenzae* but growing readily on plain agar (21) suggests that the organism may have been of the genus *Pasteurella*. In those studies where endogenous bacteria multiplying in murine lungs after virus infection were identified, the organisms reported were *Pasteurella pseudotuberculosis* (36), *Haemophilus influenzae-murium* (36), and *Pasteurella pneumotropica* (33).

The data presented in this investigation have a clear clinical significance. The occurrence of a synergistic effect between these two natural infectious agents in the mouse may well reflect the human condition where secondary bacterial complications with pathogens such as *S. aureus*, *Diplococcus pneumoniae*, *H. influenzae*, and other gram-negative organisms are frequently seen subsequent to primary viral infections. Furthermore, the appearance and proliferation of an endogenous *Pasteurella* in the lung parenchyma of Sendai virus-infected mice indicates that organisms which are part of the upper respiratory tract flora can be aspirated to and multiply in the lower respiratory tract during concomitant virus infection.

It is interesting to speculate that if such a synergistic effect is operative in human infection, the development of attenuated viral vaccines, administered by aerosol, might also provide an additional benefit by decreasing the incidence of secondary bacterial infections.

**ACKNOWLEDGMENTS**

This investigation was supported in part by Public Health Service training grants 5-TI-GM-822 and 9-TI-AI-00296 from the National Institute of General Medical Sciences and the National Institute of Allergy and Infectious Diseases, respectively. A Public Health Service research grant (AI-01299) was also awarded from the National Institute of Allergy and Infectious Diseases along with a NASA grant (50-002-078) and a Public Health Service career development award (K3-AI-21,758) from the National Institute of Allergy and Infectious Diseases. We thank Carlyn L. Tucker, Pathologist, Wisconsin State Laboratory of Hygiene, for confirming our histologic observations, and Peter B. Smith, Chief, Bacterial Reference Unit, NCDC, Atlanta, Ga., for confirming our identification of *Pasteurella* isolates.

**LITERATURE CITED**

2. Beard, C. W., and B. C. Easterday. 1965. An aerosol...


