Persistence of *Diplococcus pneumoniae* after Influenza Virus Infection in *Macaca mulatta*

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Exposure of monkeys to aerosols of influenza virus followed later by aerosols of *Diplococcus pneumoniae* resulted in persistence of the bacteria in the upper respiratory tract for an average of 28 days. Examination of selected tissues for pneumococci revealed that pneumococci were widely disseminated throughout the respiratory tract at 3 and 7 days after exposure, but were cleared from lower respiratory tissues and were found mainly in tonsils, oropharynx, and posterior nasopharynx after 14 days.

Bacterial pneumonia after influenza virus infection was recognized as an important medical problem long before the etiology of influenza was identified, and this sequence of infections still constitutes a serious disease syndrome (14). It is well established that the organisms isolated most frequently from patients with postinfluenzal pneumonia are *Diplococcus pneumoniae* and *Staphylococcus aureus* (3, 7, 15, 16, 18). In spite of the importance of this disease syndrome, most published reports have dealt with clinical findings of patients involved in epidemics rather than with experiments dealing with laboratory animals. This paucity of experimental data may be attributed to the lack of a suitable animal model and to the difficulty of employing aerosol exposures to establish sequential infection with influenza virus and gram-positive bacteria. Some investigators, however, have utilized influenza virus to study sequential infection in laboratory mice. Francis reported that synergism occurred between influenza virus and *Haemophilus influenzae* after intranasal instillation only if the bacteria were administered at least 24 h after the virus (5). Gerone and co-workers (6) reported that aerosol-induced pneumococcal infection after influenza virus exposure to aerosols depressed viral antibody formation and terminated in fatal pneumococcal pneumonia. Synergistic activity of inhaled influenza virus and pneumococci or streptococci has also been described by others (8, 9; H. N. Carlisle and N. P. Hudson, J. Bacteriol. 53:503-504 [Abstr.], 1947). A possible mechanism for the response of mice to sequential infection was postulated by Sawyer (17), who reported that phagocytosis of pneumococci by polymorphonuclear leukocytes and alveolar macrophages is inhibited by influenza virus.

Use of subhuman primates for similar studies has been limited, primarily because conditions for establishing clinical influenza in monkeys are poorly understood. Several decades ago, however, Wilson and co-workers (19) described clinical illness in 5 of 11 monkeys that were challenged intranasally with *Streptococcus hemolyticus* 4 to 17 days after receiving the PR-8 strain of influenza virus, and suggested that the prior exposure to influenza caused alterations in both humoral and cellular defense mechanisms.

We have taken advantage of improved capability for control of dosage and particle size of aerosols of microorganisms to examine various facets of the effect of viral infection on the persistence of *D. pneumoniae* in various tissues of primates.

**MATERIALS AND METHODS**

**Influenza virus.** The Aichi/2/68 strain of type A (H3N2) influenza virus was propagated by allantoic cavity inoculation of 10- to 13-day-old embryonated eggs. Procedures for growth, harvest, and storage have been described previously (2). An aerosol challenge dose consisted of approximately 10^4 mean egg infectious doses.

**D. pneumoniae.** Type 1 *D. pneumoniae* (ATCC 6301) was grown in 5 ml of brain heart infusion broth supplemented with 10% normal rabbit serum and 0.05 ml of fresh sheep blood. Prior to preparation of a challenge suspension, the virulence of the organisms was enhanced by rat passage. Rats were inoculated intraperitoneally with 0.5 ml of selected dilutions of pneumococcal culture; after 72 h, heart blood from sick rats was injected into a second group of rats, and, after a third passage, heart blood from moribund rats was used as the inoculum for broth cultures. These cultures were incubated for 18 h at 37 C, examined for purity, and subcultured to fresh tubes of broth. The subcultures were incubated for 4 h and then were used
without further treatment. Viable counts of bacterial suspensions were determined by spreading portions of appropriate dilutions on blood agar base plates supplemented with 5% sheep blood. Unless otherwise stated, an aerosol challenge dose consisted of 10⁶ pneumococci.

**Aerosol dissemination and particle size estimation.** The procedures and apparatus employed for aerosol dissemination and particle size estimation have been described previously (2). The Collison atomizer was employed for dissemination of small-particle aerosols of influenza virus (median diameter, 2.0 μm), and the Dautrebande nebulizer (4) was substituted to disseminate pneumococcal aerosols (median diameter, 2.4 μm) because the viscosity of suspensions of pneumococci was sufficiently high to produce aerosol particles of 3.5-μm diameter. The size distribution of the aerosol particles was estimated by the method of Malligo and Idoine (13).

**Experimental animals.** *Macaca mulatta* (rhesus monkeys), weighing from 2.5 to 4.5 kg, were conditioned for 60 days before experimental use. They were allowed free access to water and commercial monkey chow after sampling procedures were completed each morning. Methods for aerosol exposure, estimation of number of organisms inhaled, and collection of blood and nasopharyngeal samples have been described previously (2).

**Serological techniques.** Antinfluenza hemagglutination inhibition titers were measured with guinea pig erythrocytes in microtiter plates, and immunoglobulin determinations were performed by the Mancini technique (2).

A modification of the passive hemagglutination test described by Ammann and Pelger (1) was employed to measure antibody to type 1 *D. pneumoniae*. Pooled rhesus monkey erythrocytes, substituted for human type O cells, were complexed to purified type 1 pneumococcal polysaccharide with chromic chloride.

**Organism isolation.** Mouse inoculation was employed to detect the presence of *D. pneumoniae* in nasopharyngeal washings and tissue samples. A volume (0.5 ml) of each wash was inoculated into a traperitorn sample of each of five mice; 0.1 ml of heart blood from mice that died or were moribund within 72 h was spread on a blood agar plate. After an optochin disk (Difco Laboratories, Detroit, Mich.) was placed on the surface, the plate was incubated for 24 h at 37 C. The presence of pneumococci was confirmed by characteristic growth, alpha hemolysis, and by a zone of inhibition around the disk.

Influenza virus was isolated by inoculation of embryonated eggs with nasal washings that had been incubated for 1 h at room temperature with 100 U of penicillin and 100 μg of streptomycin per ml. After 48 h of incubation at 35 C, the presence of the virus in allantoic fluid was determined by standard hemagglutination titrations.

**Radiographic examination.** Where indicated, anterior-posterior and left-lateral radiographs of the thorax were obtained from monkeys that had been anesthetized with ketamine hydrochloride (Ketaset; Bristol Laboratories, Syracuse, N.Y.).

**Collection and processing to tissue samples.** Monkeys were sacrificed by intravenous inoculation of 1.2 g of pentobarbital. The chest was opened aseptically, and sections of the diaphragmatic lobe of the left lung, secondary bronchus adjacent to it, a bronchial lymph node adjacent to the left main bronchus, a piece of the middle third of the trachea, larynx, oropharynx, right tonsil, and posterior and anterior nasopharynx were excised and placed in 5.0 ml of heart infusion broth. Nasal mucosa was obtained with a stiff test tube brush that was immersed in broth. An effort was made to remove a sample of the same size from each monkey. The right lung was perfused with formalin, and a sample equivalent to each described above was removed and placed in formalin for histopathological examination. The tissues were ground in Ten-Broeck grinders, and 0.5 ml of each was inoculated into five mice for isolation of pneumococci as previously described. The number of mice dying in each group, therefore, gave a rough approximation of the number of bacteria contained in each sample.

**RESULTS**

**Preliminary experiments.** Twelve monkeys were exposed to aerosols of influenza virus; one group of three monkeys served as virus infection controls, and three groups of three monkeys each were exposed to aerosols of pneumococci 3, 7, or 10 days, respectively, after exposure to the virus. Three additional monkeys served as pneumococcal infection controls and two monkeys received no treatment. On the 5th and 6th days prior to virus exposure and from day 1 (24 h after exposure) through day 9, rectal temperatures were recorded, blood samples were obtained for serology, total and differential leukocyte counts, and hematocrit, and nasopharyngeal washings were collected for immunoglobulin A (IgA) and IgG determinations and for isolation of virus and *D. pneumoniae*. Samples were obtained on days 11, 13 and 17 postexposure for immunoglobulin assay and serology, and daily for isolation of virus and bacteria, until the organisms could no longer be isolated.

Pneumococcal recovery from the 3-, 7-, and 10-day sequentially exposed groups of three monkeys each ranged from 7 to 99 days (mean of 28), whereas the bacterial recovery from the pneumococcal control group ranged only from 2 to 11 days (mean of 7). Leukocyte counts, hematocrit levels, and rectal temperatures showed no significant change.

Geometric mean values for antipneumococcal hemagglutination inhibition titers were very low, but the response of the 3-day group appeared earlier and rose to higher levels than the control group, suggesting that prior influenza virus infection either increased host responsiveness to pneumococcal antigen or resulted in...
production of an increased antigenic mass (bacteria) (Table 1). Geometric mean values for antiinfluenza hemagglutination inhibition titers, however, showed no significant differences.

Nasopharyngeal IgA responses are presented in Fig. 1. By analysis of variance, no significant differences in nasopharyngeal IgA values were evidenced between groups with viral exposure and, likewise, between groups with no viral exposure (pneumococcal and sham control groups). Peak values for influenza control and sequentially infected groups were significantly higher ($P < 0.01$) than those for sham and pneumococcal control groups. Thus, exposure to influenza virus resulted in increased IgA production, whereas exposure to pneumococci had no effect on IgA production. Indirect evidence that IgA was of secretory rather than serum origin was provided by the observation that the samples contained no detectable IgG.

**Additional experiments, site of persistence.** Since preexposure to influenza virus appeared to establish a condition favorable for prolonged persistence of pneumococci in nasopharyngeal tissues, an experiment was performed to determine the site of residence of the bacteria. Groups of eight monkeys each were exposed (i) to $10^4$ mean egg infectious doses of influenza virus followed 3 days later by exposure to $10^4 D. pneumoniae$, (ii) to pneumococci alone, (iii) to influenza virus alone, or (iv) to no treatment. At 7, 14, 21 and 28 days after pneumococcal exposure, two monkeys from each group were reexamined radiographically; blood for serological studies was obtained, the monkeys were sacrificed, and tissues were collected for culture and histological examination.

**Table 1. Development of hemagglutination response to type 1 pneumococcal polysaccharide after aerosol exposure of monkeys to influenza virus followed by $10^4 D. pneumoniae$**

<table>
<thead>
<tr>
<th>Treatment with $D. pneumoniae$</th>
<th>Reciprocal geometric mean titer at indicated day after pneumococcal exposure$^c$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>3 days after influenza virus</td>
<td>2 (1.7–2.7)$^a$</td>
</tr>
<tr>
<td>7 days after influenza virus</td>
<td>2 (1.7–2.7)</td>
</tr>
<tr>
<td>10 days after influenza virus</td>
<td>0$^b$</td>
</tr>
<tr>
<td>Controls</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ All preexposure titrations were less than 1:2.
$^b$ Values in parentheses indicate geometric standard error ranges.
$^c$ Titer was less than 1:2.
TABLE 2. Recovery of *D. pneumoniae* from selected upper respiratory tissues

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of monkeys</th>
<th>No. of dead mice/no. injected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7°</td>
<td>14</td>
</tr>
<tr>
<td><strong>Control monkeys</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasal mucosa</td>
<td>2</td>
<td>0/8</td>
</tr>
<tr>
<td>Anterior nasopharynx</td>
<td>3</td>
<td>5/15</td>
</tr>
<tr>
<td>Posterior nasopharynx</td>
<td>3</td>
<td>14/15</td>
</tr>
<tr>
<td><strong>Sequentially infected monkeys</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasal mucosa</td>
<td>2</td>
<td>5/8</td>
</tr>
<tr>
<td>Anterior nasopharynx</td>
<td>3</td>
<td>7/15</td>
</tr>
<tr>
<td>Posterior nasopharynx</td>
<td>3</td>
<td>14/15</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>5</td>
<td>20/23</td>
</tr>
<tr>
<td>Tonsil</td>
<td>5</td>
<td>21/23</td>
</tr>
</tbody>
</table>

*a* Vertical bars show statistically significant difference between indicated tissues (Fisher exact test).

*b* Days after exposure.

*c* Monkeys exposed to *D. pneumoniae* only.

*d* Monkeys exposed to virus followed by *D. pneumoniae*. 
as previously described. The experiment was replicated with three monkeys at each time for each treatment. Samples of anterior and posterior nasopharynx were substituted for the nasal mucosa obtained in the first experiment to determine the site of replication more precisely. Statistical analysis indicated that the results of the two experiments could be combined (Table 2). Pneumococci were never isolated from sham control monkeys and, except for a few bacteria in 7-day samples, rarely were recovered from tissues located below the oropharynx in any of the monkeys. Recovery of pneumococci from upper respiratory tissues was greater in sequentially infected monkeys than in the controls \( (P < 0.001, \) Fisher exact test) over all time periods. Incidence of recovery of bacteria at 28 days was highest from the tonsils, oropharynx, and posterior nasopharynx. The greatest significant difference between sequentially infected and control monkeys was obtained from the tonsils.

Radiographic and histological examinations revealed no changes attributable to the experimental procedures. Thus, although clinical pneumonia failed to develop, pneumococci persisted in the tissues of the upper respiratory tract.

**Pattern of growth.** Since prior influenza virus infection played a significant role in the persistence of pneumococci in tonsils and throat, an additional experiment was performed to elucidate further the pattern of infection. Twelve monkeys were exposed to \( 10^4 \) mean egg infectious doses of influenza virus followed 3 days later by exposure to \( 10^5 \) *D. pneumoniae*. Three monkeys were sacrificed 3 days after *D. pneumoniae* exposure, two at 7 days, three at 14, and two each at 21 and 28 days. Recovery of pneumococci from tissues in the upper and lower respiratory tract are summarized in Table 3. At 3 days, significant numbers of pneumococci were scattered throughout the respiratory tract, although the lung and bronchial lymph node contained only small numbers. At 7 days, pneumococci were isolated from every tissue tested. At 14 days, bacteria had disappeared from areas below the larynx and by 21 days were confined to tonsilar, oropharyngeal, and posterior nasopharyngeal areas. As in the preceding trial, pneumococci were isolated for the longest time and in greatest numbers from tonsilar tissue.

**DISCUSSION**

Sequential exposure with small-particle aerosols of influenza virus followed by exposure to *D. pneumoniae* failed to elicit clinical bacterial pneumonia. Nevertheless, the initial virus infection did alter the host environment sufficiently to prolong the persistence of pneumococci in the upper respiratory tract. If our model is representative of what might occur naturally in man, it is suggested that the pneumococci that gain entrance after viral infection persist in the upper respiratory tract and become an autogenous source of bacteria for future illness should the host fail to develop a specific immune response to the initial bacterial "colonization." In this regard, it is significant that monkeys failed to respond with an increase in IgA after exposure to *D. pneumoniae* alone. Also, the serological response to *D. pneumoniae* (passive hemagglutination test) was of a low order. Failure to develop marked immune re-

**Table 3. Persistence of pneumococci in selected tissues from twelve monkeys after sequential exposure**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Occurrence and relative frequency of isolation</th>
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<tbody>
<tr>
<td></td>
<td>3°</td>
</tr>
<tr>
<td>Lower respiratory tract</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>TR</td>
</tr>
<tr>
<td>Bronchus</td>
<td>+</td>
</tr>
<tr>
<td>Bronchial lymph node</td>
<td>TR</td>
</tr>
<tr>
<td>Trachea</td>
<td>+</td>
</tr>
<tr>
<td>Upper respiratory tract</td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>+</td>
</tr>
<tr>
<td>Tonsil</td>
<td>+</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>+</td>
</tr>
<tr>
<td>Posterior nasopharynx</td>
<td>+</td>
</tr>
<tr>
<td>Anterior nasopharynx</td>
<td>+</td>
</tr>
</tbody>
</table>

* Trace (TR), <25% of isolation frequency; +, 25 to 40% of isolation frequency; ++, 50 to 75% of isolation frequency; ++++, >75% of isolation frequency; —, no viable pneumococci isolated.

* Days after pneumococcal exposure. Three monkeys were sacrificed on days 3 and 14; two monkeys were sacrificed on days 7, 21, and 28.
sponse to upper respiratory persistence has been theo-

erized as a contributing factor to pneumonia in

humans by MacLeod (12), who asserted that the

chance of developing bacterial pneumonia during

viral epidemics depends in great part upon

whether an individual is a carrier of a virulent type of D. pneumoniae to which he is

not immune. The importance of an autogenous

source of bacteria for the development of pneu-

monia has recently been emphasized by Joha-

son et al. (10), who have shown that pneumonia
due to gram-negative bacilli in seriously ill

patients in hospitals is caused by aspiration of

bacteria from the pharynx.

Our most important observation is the per-
sistence of bacteria in the upper respiratory

tract. The occurrence of this reaction, if it

occurred in humans after mild respiratory virus

infection, could constitute one means by which a

carrier state may be established. In contrast,

these studies failed to yield supportive evidence

for the commonly accepted explanation for

development of pneumococcal pneumonia sub-

sequent to severe influenza in human pa-

tients, whereas it has been postulated that

influenza virus infection causes necrosis of the

tracheo-bronchial epithelium and edema (8, 9,

11), and thereby provides a site in which bac-

teria can multiply relatively undisturbed by

host clearance mechanisms. We cannot elimi-

nate the possibility that such a reaction oc-

curred in the upper respiratory tract, but it was

not evident histologically at any time of sam-

pling. It is equally possible, however, that some

other mechanism, directly or indirectly, is re-

sponsible for alteration of the microenviron-

ment of the tonsils and oropharynx in such a

way as to favor bacterial persistence.

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antibody to pneumococcal polysaccharides with

chromic chloride-treated human red blood cells and


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