Respiratory Diseases in Cyclophosphamide-Treated Mice

I. Increased Virulence of Mycoplasma pulmonis

S. H. SINGER, M. FORD, AND R. L. KIRSCHSTEIN

Laboratory of Pathology, Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 11 February 1972

Mice infected intranasally with Mycoplasma pulmonis were treated with cyclophosphamide, a potent immunologic suppressor. In place of a chronic smouldering infection with little mortality (2%), a rapidly lethal infection with high mortality (66%) was produced. M. pulmonis was able to be isolated from several organs during the course of the unmodified infection. In the infected animals treated with cyclophosphamide, dissemination occurred earlier, and higher titers of mycoplasma were found. Reconstitution experiments with spleen cells from previously infected animals reversed the effect of cyclophosphamide, indicating that immunity plays an important role in containment of the infection and eventual recovery.

Mycoplasma pulmonis infection of mouse lung is an interesting entity in that the organism can induce a prolonged and chronic infection of the respiratory passages with persistence of mycoplasma months after infection. Gross and microscopic changes of pneumonia can be found in the lungs of infected mice, and yet with certain strains mortality, when present, remains extremely low (4). We attempted to determine those factors which are involved in the protection and eventual recovery from this disease and decided to use cyclophosphamide, a potent immunosuppressive agent as an investigative tool. Treatment of animals with cyclophosphamide has been reported to lead to enhanced replication of infecting organisms or death, or both, following exposure to bacteria (5, 10), mycobacterium (6), trypansomes (2), and viruses (1, 3, 7–9). It was, therefore, of further interest to determine what the effects on a mycoplasma infection would be. Our results indicate that a chronic smoldering mycoplasma infection can become a rapidly virulent one in immunosuppressed mice and that host immunity plays an important role in the early containment of the mycoplasma infection to the lung and in protection against the potential lethal effects of the organism.

MATERIALS AND METHODS

Animals. Groups of at least 10 general purpose Swiss female mice with a median weight of 20 g obtained from the Rodent and Rabbit Production Section, Laboratory Aids Branch, National Institutes of Health (NIH), were used in these experiments.

Inoculations and Inocula. A pool of the Ash strain of M. pulmonis was prepared in broth culture. Infection was initiated by instilling 1 minum (0.06 ml) of an M. pulmonis suspension with a titer of $1.6 \times 10^9$ per ml into the external nares of lightly anesthetized mice. Using this method of inoculation, we were able to infect the lungs successfully and further to recover organisms from the infected lungs of 100% of the treated mice. Simultaneous with the initiation of infection on day 0, each mouse was inoculated intraperitoneally with 0.1 ml of either pyrogen-free saline or with cyclophosphamide at a concentration of 80 $\mu$g per g of body weight. The intraperitoneal injections were given once daily for a total of four inoculations.

Spleen cells used in reconstitution-type experiments were obtained from animals which had been intranasally infected with M. pulmonis 9 to 10 days previously. Assay indicated that there were no viable mycoplasmas or metabolic inhibition test (MIT) antibodies in the suspensions at time of use.

Mycoplasma titrations. Mycoplasma was titrated by observing the color change in PPLO broth w/o CV (Difco Laboratories, Detroit, Mich.) to which 0.004% cresol red, dextrose, glutamine, vitamins, 20% horse serum, 10% yeast extract, 0.025% thallium acetate, and penicillin at 1,000 units/ml were added. The broth cultures were incubated for 1 week, and the end point was read as the last tube to show a demonstrable color change from control tubes. Isolation of mycoplasma from selected organs was attempted by first homogenizing the organ as a 10% suspension by weight in Eagle minimal essential medium and then assaying in the above-described
medium. Mycoplasma isolates were identified as *M. pulmonis* by a fluorescent-antibody technique.

A metabolic inhibition test for antibody was performed by incubating the test material with 100 colony-forming units of *M. pulmonis*. Antibody titer was read as the last dilution preventing a color change by the mycoplasma in the above described medium. As a positive control, the NIH Research Reagent *M. pulmonis* (Ash) antiserum was used. This titered 1:256 in our assay.

**RESULTS**

With the strain and dose of mycoplasma used, a chronic infection with a relatively nonlethal disease was readily established. Gross and microscopic evidence of lung disease was seen at 48 to 72 hr postinfection and persisted for at least 1 week. *M. pulmonis* could be isolated from the lungs in high titer during this period (see Table 2). Mortality after infection was low and averaged 2% in a series of experiments involving a total of 50 mice even when the animals were observed for periods ranging up to 3 weeks postinfection.

The cyclophosphamide injections were well tolerated, with little mortality at the described dose (averaging 4% in our series). As a measurement of the action of cyclophosphamide, peripheral white blood counts (WBC) were performed at selected intervals. Before treatment these averaged 2,600 WBC per mm³. One day following administration of the fourth and last cyclophosphamide dose, the average peripheral WBC count was 418 per mm³. On day 7 (168 hr postinfection and post first cyclophosphamide dose), the WBC count was rising and averaged 1,400 per mm³. Based on the results of these counts, experiments were terminated on day 7 postinfection.

Cyclophosphamide-treated animals infected with *M. pulmonis* had a significantly higher mortality rate (average 66%) than animals treated with cyclophosphamide only (4%) or animals infected only with *M. pulmonis* (2%). This is shown in Table 1, in which the cumulative mortality figures from five separate experiments are recorded.

Organs were removed from infected, cyclophosphamide-treated and untreated mice at selected intervals to study the course of infection in these animals. As can be seen from Table 2, several trends were apparent. The *M. pulmonis* infection was not limited to the lungs even in the mice which were not treated with cyclophosphamide, and at 96 hr postinfection, *M. pulmonis* was found not only in the lung tissue but also in liver, spleen, and brain. In the cyclophosphamide-treated animals, however, this spread of infection occurs earlier since the organism was isolated from multiple organs at 48 hr. Mycoplasma titers were consistently higher in the lungs of cyclophosphamide-treated mice with the greatest difference (3.1 logs) occurring 96 hr postinfection. Persistence of higher titers was also noted in the livers and brains of cyclophosphamide-treated, infected mice at 168 hr postinfection.

The role of circulating neutralizing antibody in this infection during the 7-day observation period was investigated by using the MIT. No such antibody was able to be detected in the sera of infected animals.

To explore further the role of immunity and to rule out the possibility that mortality in the *M. pulmonis*-cyclophosphamide-treated animals was due to combined toxicity of the cyclophosphamide and *M. pulmonis*, reconstitution experiments were performed. Fifty million spleen cells from previously infected animals were infused intravenously into each of a group of cyclophosphamide-treated, *M. pulmonis*-infected animals on the second day (48 hr) postinfection. The results

---

### Table 1. Cumulative mortality of *Mycoplasma pulmonis* in cyclophosphamide-treated and untreated mice

<table>
<thead>
<tr>
<th>Expt</th>
<th><em>M. pulmonis</em> alone (%)</th>
<th>Cyclophosphamide alone (%)</th>
<th><em>M. pulmonis</em> + cyclophosphamide (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>Avg</td>
<td>2</td>
<td>4</td>
<td>66</td>
</tr>
</tbody>
</table>

* Cumulative mortality 7 days (168 hr) post-intranasal infection with *M. pulmonis*. Each experiment represents a minimum of 10 animals per group.

### Table 2. *Mycoplasma* titers from cyclophosphamide-treated and untreated mice

<table>
<thead>
<tr>
<th>Time post-infection (hr)</th>
<th>Lung</th>
<th>Liver</th>
<th>Spleen</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>5.4</td>
<td>6.0</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>96</td>
<td>3.9</td>
<td>7.0</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>168</td>
<td>5.8</td>
<td>6.7</td>
<td>1.9</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Titers expressed as log₁₀ *M. pulmonis* per ml of a 10% organ suspension. Co, untreated; Cycloph., cyclophosphamide-treated.
spleen cells from previously uninfected animals. This most probably would not be the case if simple toxicity were involved. These results also argue against the possibility that activation of a latent agent or superinfection was responsible for the deaths of the animals, since in that case the effect of giving spleen cells from animals previously or not previously exposed to *M. pulmonis* should be the same.

The differences in the titers of mycoplasma recoverable from the lungs of the two groups at first suggested that the number of organisms in the lungs themselves may be a critical factor. However, gross and microscopic examinations of the lungs from the two groups were similar and failed to explain the marked increase in mortality in the cyclophosphamide-infected animals. The spread of mycoplasma is accelerated in the cyclophosphamide-treated animals. Although pathological changes were found in the lungs only and not in the other organs, and although dissemination occurs in the unmodified infection also, the earlier spread in cyclophosphamide-treated animals may be critical and may, in some way, contribute to the increased mortality.

We were unable to demonstrate circulating antibody during the first 7 days postinfection by using the MIT. It is always possible that other methods may prove to be more sensitive in the detection of humoral antibody during this early postinfection time period. Also, since the infusion of spleen cells used in the reconstitution experiments contains cells potentially capable of immunoglobulin production as well as sensitized lymphocytes, it remains a possibility that humoral antibody as well as cell-mediated immunity plays a role in the containment of the mycoplasma infection. Further experiments are in progress to elucidate this.

Previous reports have indicated that cyclophosphamide-treated mice which are infected with various bacteria succumb more readily than infected, untreated mice (5, 10); clinicians, utilizing immunosuppressives in clinical practice, also have become aware of this. Our data indicate that similar treatment can also enhance the lethality of mycoplasma infection in mice. It is possible, therefore, that mycoplasmas may be responsible for some of the cases of overwhelming infections in immunosuppressed human hosts. Awareness of this may lead to increased detection of such instances and, thus, to specific antibiotic therapy.

**DISCUSSION**

Our results indicate that cyclophosphamide treatment of mice can transform a smoldering, chronic mycoplasma infection into a lethal one. This does not appear to be a result of the combined toxicity of the infection and the cyclophosphamide since the mortality of the mycoplasma-infected, cyclophosphamide-treated mice is much higher (66%) than a summation of the mortalities of *M. pulmonis*-treated (2%) or cyclophosphamide-treated (4%) animals alone or any multiple thereof. Of more significance, in reconstitution experiments, reversal of this mortality was accomplished with spleen cells from animals previously infected with *M. pulmonis* but not with...