Effect of Cyclophosphamide on the Growth of *Rickettsia sennetsu* in Experimentally Infected Mice

NOBUYOSHI TACHIBANA* AND YUZURU KOBAYASHI

First Department of Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan

Received for publication 2 May 1975

The growth rate of *Rickettsia sennetsu*, the etiological agent of sennetsu rickettsiosis, a special type of infectious mononucleosis found in western Japan, is very low when the ordinary experimental methods used for other members of the rickettsiae are employed. Attempts have been made to increase its growth rate in cells and animals. By treating mice with cyclophosphamide, considerable enhancement of growth of the rickettsia was observed. In the drug-treated mice, infective titers expressed as mean lethal dose in the spleens reached 10⁴·⁴ in animals treated by the intraperitoneal route three times at 5-day intervals with 0·33 mg/g (body weight). Infective titers in drug-treated mice were more than 100-fold above those of the infected non-drug-treated mice. In peritoneal cells of drug-treated mice rickettsial particles were observed in great abundance. The numbers of cells containing rickettsiae almost paralleled the infective titers of the spleens in each group of mice. Lymph nodes and spleens of the drug-treated mice diminished in size during infection.

Infectious mononucleosis, which is widely distributed, is characterized by fever, general lymph node enlargement, and absolute increase in normal and atypical lymphocytes. In western Japan the disease has been recognized in several districts for many years.

In 1953, Misao and Kobayashi isolated an agent from blood, lymph node, and bone marrow fluid of a patient in Fukuoka who showed the typical symptoms of infectious mononucleosis (9). The organism was confirmed to be the etiological agent of the disease by experimental infection in human volunteers (9). Although the natural cycle of the agent has not been fully established, the agent has the general morphological and biological properties of rickettsiae. The agent was therefore named *Rickettsia sennetsu* in 1956 (10–12).

The disease caused by *R. sennetsu* shows the same clinical and pathological symptoms as infectious mononucleosis in other countries (5, 14, 16); however, Kobayashi observed that the sera from patients in the United States with infectious mononucleosis gave a negative reaction when tested against *R. sennetsu* by the immunofluorescent antibody test (8). This suggested that infectious mononucleosis is not a disease but a syndrome. Thus, the disease caused by *R. sennetsu* has been called "sennetsu rickettsiosis" or infectious mononucleosis in western Japan, differentiating it from infectious mononucleosis of unknown etiology in other parts of Japan and other countries (8). Further studies on *R. sennetsu*, such as purification of rickettsiae or preparation of rickettsial antigen for immunological analysis of rickettsia, are in progress. The essential problem in the study has been to obtain enough rickettsiae, owing to the slow growth rate of the organism in developing hen eggs, cultured cells, or ordinary laboratory animals (9, 17).

The present study was initiated to elucidate the influence of cyclophosphamide, a carcino- static or immunosuppressive drug, on the growth of *R. sennetsu* in experimentally infected mice. Drug treatment significantly enhanced the growth of rickettsia in the animals.

**MATERIALS AND METHODS**

**R. sennetsu.** The Miyayama strain, prototype of *R. sennetsu*, isolated in 1953 by Misao and Kobayashi (9) and kept in this laboratory by passage in mice over 300 times, was used. For preparation of the inoculum, spleens of infected mice were obtained on day 12 of infection and homogenized in a Waring blender for 1 min with 10 or 20 times the volume of phosphate-glutamate-sucrose solution (PGS) (3). The homogenate was centrifuged at 1,000 rpm for 5 min, and the supernatant was adjusted with PGS to make a 10⁻² suspension. The suspension (0·2 ml) was inoculated into mice by the intraperitoneal route.

**Mice.** Ten-week-old mice of the dd strain raised in this laboratory were used. In the experiment, each group consisted of 10 mice.

**Cyclophosphamide.** Endoxan (Shionogi & Co.,
Ltd.), 100 mg per vial, was used. The drug was dissolved in 3.0 ml (33.0 mg/ml) or 6.0 ml (16.5 mg/ml) of physiological saline solution just before injection, and 0.01 ml of the solution per g of body weight was given by intraperitoneal injection according to the schedules described below.

**Administration of the drug.** Cyclophosphamide was given to group 1 seven days after infection to make the effect of the drug coincide with the actively propagating phase of the rickettsiae. In group 2, mice were given the drug 2 days before and 7 days after infection to test whether the effect was enhanced by double doses given both in the early and late stages. Mice in group 4 were given the drug 2 days before and 7 and 9 days after infection, and the amount was reduced to one-half that given the former groups. In groups 3 and 5, mice received the drug three times at 5-day intervals and were tested to make the effect continue throughout the whole period of the rickettsial growth (Table 1).

**Determination of infective titer of the spleens of mice infected with R. sennetsu.** General findings and change of weight of both drug-treated and control mice were observed every day. Twelve days after inoculation of the rickettsia, when all infected mice were severely sick, four mice from each group were sacrificed. The spleens were removed and homogenized in a Waring blender in PGS to make a 10% suspension. The supernatant was drawn off and diluted 10^{-3} with PGS. Further 10-fold serial dilutions were prepared in the same diluent from 10^{-3} through 10^{-4}, and 0.2 ml of each dilution was inoculated intraperitoneally into each of three mice. These mice were observed for 6 weeks and the mean lethal dose was calculated by the method of Kärber (13).

**Microscope examination of rickettsiae in peritoneal smears from infected mice.** Peritoneal smears from both drug-treated and control mice were Giemsa stained and observed under a light microscope. From 1,000 to 2,000 peritoneal cells were observed on each smear, neutrophilic leukocytes excepted, and the number of cells containing rickettsial particles was calculated.

**RESULTS**

**Effect of cyclophosphamide on normal mice.** Since the cyclophosphamide given in this study was a relatively large dose, it seemed necessary to test whether the drug treatment itself had any effect on mice. For this purpose, three or four mice in each group were given the drug alone as the drug control and observed for general symptomatology and daily change of body weight.

The result of one of the three experiments is presented in Fig. 1. The body weight of normal control mice without any treatment increased gradually to 8.7% above the initial weight after 14 days. On the other hand, weight in the drug-treated groups decreased without exception. In group 1, weight of the mice showed a decrease of 16.0% on day 3 of drug administration and slightly recovered thereafter. In group

**Table 1. Schedule of inoculation of R. sennetsu and cyclophosphamide**

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CP</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CP</td>
<td></td>
<td>CP</td>
<td></td>
<td>CP</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>CP</td>
<td>R</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td>CP</td>
<td></td>
<td>CP</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
</tr>
<tr>
<td>3</td>
<td>cp</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td></td>
<td></td>
<td>CP</td>
<td></td>
<td>CP</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
</tr>
<tr>
<td>4</td>
<td>cp</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td></td>
<td>CP</td>
<td></td>
<td>CP</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
<td>cp</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* R, R. sennetsu, Miyayama strain; CP, cyclophosphamide, 0.33 mg/g (body weight); cp, cyclophosphamide, 0.165 mg (body weight); +, sacrificed.
2, weight loss after the first dose was almost the same as that of group 1 and was more conspicuous after the second dose. In group 3, weight loss was more prominent, and loss of 20.0% of the weight was observed on day 8, when one mouse died. The mice in groups 4 and 5 showed almost the same change as those in group 3. In general, ruffled fur and weakness were observed in all mice that lost weight. Thus, 0.33 mg of cyclophosphamide per g of body weight was toxic to all mice but did not make the experiments impossible.

**Infective titers of the spleens from infected mice.** The results obtained in experiments 1, 2, and 3 are shown in Table 2. Infective titers, expressed as mean lethal dose, of infected control mice were $10^{4.9}$ in experiment 1 and $10^{4.2}$ in both experiments 2 and 3. Infective titers of group 1 were $10^{4.1}$, $10^{7.4}$, and $10^{7.2}$, and those of group 2 were $10^{4.6}$, $10^{7.2}$, and $10^{4.3}$, respectively. Treatment with a single drug dose was effective, but an additional drug administration appeared to increase the effect. The titers of group 4 were $10^{8.3}$ in experiment 1 and $10^{7.4}$ in both experiments 2 and 3. Less variation in titers was observed in this group. The titers of groups 3 and 5 were both $10^{4.3}$ and more than 100-fold above those in the control animals.

**Rickettsial particles in peritoneal cells from infected mice.** Peritoneal smears from all mice, in both drug-treated and infected control groups, were examined microscopically. It was not easy to find rickettsial particles in smears from the infected control mice. In contrast, infected cells were quite numerous in the drug-treated groups, and the numbers paralleled the increase in infective titers of spleens. The findings of experiment 3 are presented in Fig. 2. In the control group, the percentage of cells with rickettsiae was only 1.0%, and the number of particles in a cell was mostly less than 10. On the other hand, in groups 3 and 5 more than 35.0% were rickettsiae containing, and one-third of such rickettsiae-containing cells were filled with particles in the cytoplasm (Fig. 3). Although in other groups both the percentage of infected cell and numbers of particles in a cell were less than in the two groups mentioned above, there was generally an increase over the controls.

### Table 2. Infective titers of the spleens from mice inoculated with R. sennetsu and cyclophosphamide

<table>
<thead>
<tr>
<th>Group</th>
<th>Infective titers a</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>8.1 (2.3) b</td>
<td>7.8 (1.6)</td>
<td>7.2 (1.0)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8.5 (2.6)</td>
<td>7.2 (1.0)</td>
<td>8.5 (2.3)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8.3 (2.4)</td>
<td>7.8 (1.6)</td>
<td>7.5 (1.6)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>8.5 (2.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>5.9</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>5.9</td>
<td>6.2</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* Expressed as log 10 mean lethal dose.
* Numbers in parentheses indicate difference in titers from the control.

**DISCUSSION**

The effect of cyclophosphamide on mice experimentally infected with *R. sennetsu* was investigated. It was demonstrated that the growth of the rickettsiae in mice treated with cyclophosphamide was significantly enhanced.

As previously reported, it is difficult to produce large numbers of rickettsiae by using yolk sac from hen eggs or cultured cells such as HeLa or L cells (7, 17). Our efforts have been concentrated on finding conditions which enhance the growth of *R. sennetsu* in host cells or animals.

Among laboratory animals, mice are the most susceptible to *R. sennetsu* (11). Infected mice show characteristic signs of enlargement of lymph nodes and splenomegaly and finally die (11).

Cyclophosphamide is an alkylating agent used as a carcinostatic or immunosuppressant...
Thus, two rickettsiae, than other in the outcome of effectiveness for the drug. By such alterations the drug suppresses immune reactions. These characteristics of the drug suggested to us that it might produce changes in the course of *R. sennetsu* infection in mice.

It was assumed that the interval of time between administrations of the drug as well as its amount were especially important in obtaining maximum effectiveness of treatment. As for time of administration, several kinds of schedules, described previously, were designed for the following reasons. *R. sennetsu* continues to grow after inoculation and shows maximum infective titer in the mouse spleen after day 7 of infection, whereas the maximum effect of the drug on the animal itself appears between 2 and 4 days after injection (1). Therefore it is believed that the drug has different effects on the outcome of infection in mice, depending upon whether it is given early or late in the infection.

All drug-treated groups demonstrated high infective titers. Even a single dose was effective if it was given during the propagating stage of the rickettsiae, as in group 1. However, the other four groups showed higher infective titers. Thus, two or three doses of the drug given both in the early and late stages of infection were better than a single dose in obtaining high effectiveness of the drug treatment. Continuous use of the drug during the whole period of infection also brought about good results, as in groups 3 and 5. These methods seemed to be better, since they are satisfactorily applicable to rickettsial strains whose growth rates were uncertain. Doses of 0.33 and 0.165 mg/g of body weight were used and proved to be effective as long as administration followed the above schedules.

An infective titer of $10^{4.8}$ (100-fold higher than that of the controls) was considered to be the maximum growth rate of the rickettsiae in mice obtainable by this method. Spleen weight of drug-treated mice decreased to one-third or one-fourth of that of the non-drug-treated control mice.

Detection of rickettsial particles in peritoneal smears by microscopic examination appears to be a simple and reliable method of confirming the infection of mice inoculated with rickettsiae. However, it is not always easy to find rickettsial particles in peritoneal cells of mice infected with *R. sennetsu*. After drug treatment of mice inoculated with rickettsiae, large numbers of cells were infected with the organism. The rate almost paralleled the infective titers; however, groups 3 and 5 gave results superior to those obtained with other groups.

As for the toxicity of cyclophosphamide on mice, it is concluded that a drug dosage of 0.33 mg/g was toxic but did not make the experiment impossible, even though mice used as the drug control were limited in number and seemed to be somewhat uneven in weight.

Fig. 3. Peritoneal cells of group 3 mice, filled with rickettsial particles. ×1,740.
The relationship between immunological and biological changes in mice treated with cyclophosphamide and enhancement of the growth of *R. sennetsu* has yet to be explained. However, it is not likely that growth of the rickettsia is enhanced by direct effect of the drug on the organism. It is possible that the drug causes changes in the relationship between the rickettsiae and the cellular environment, allowing a marked increase in the number of organisms without greatly hastening the death of mice. Kazár and co-workers reported the enhancing effect of cyclophosphamide on experimental rickettsial infection of animals. They ascribed the effect of the drug to the suppression of both humoral and cellular immune responses (6). The mechanism may be in part due to an immunosuppressive action and in part due to nonspecific toxic action of the drug.

**ACKNOWLEDGMENT**

We wish to thank Edward S. Murray of the Department of Microbiology, Harvard School of Public Health, for reviewing this paper.

**LITERATURE CITED**