Purification of Complement-Fixing Antigens of Rickettsia sennetsu by Ether Treatment

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Soluble and particulate complement-fixing antigens of Rickettsia sennetsu were prepared from spleen suspensions of mice infected with the rickettsia and treated with cyclophosphamide. The medium used during purification of antigens, a solution consisting of equal volumes of phosphate-glutamate-sucrose buffer and veronal-buffered saline, was suitable for obtaining antigens with high titers and no anti-complementary activity. By heat treatment, it was demonstrated that the soluble antigen was heat labile and the particulate antigen was heat stable. The soluble antigen was precipitated by 80% saturation with ammonium sulfate. Cross-complement fixation tests using both soluble and particulate antigens revealed that there was no antigenic difference among strains of R. sennetsu. On the other hand, no cross-reactivity was observed between R. sennetsu and R. orientalis.

Sennetsu rickettsiosis is a disease characterized by fever, lymphadenopathy, and an increase in peripheral lymphocytes with a high proportion of atypical cells. The clinical and pathological findings were identical with those of infectious mononucleosis (8-10). The disease occurred in western Japan and was confirmed to be caused by Rickettsia sennetsu by Misao et al. (8-10).

Isolation of the rickettsia by using mice has been necessary for laboratory diagnosis of sennetsu rickettsiosis. Recently, an immunofluorescent antibody technique has become available as a specific serological reaction (3). However, the complement fixation test is an important method not only for serological diagnosis as a rickettsial disease but also for immunochromical analysis of the rickettsia.

Studies on the complement fixation test of R. sennetsu have been conducted since the discovery of the agent (4). However, the growth rate of R. sennetsu is very low in the yolk sac of hen eggs, in cultured cells, and in laboratory animals in comparison with those of other rickettsiae. Therefore, it was difficult to prepare complement-fixing antigen with high titer from these materials (4).

Treatment of mice with cyclophosphamide enhanced the growth of the rickettsia (14). This suggested that spleen suspensions of mice might be available as a source of the rickettsia for purification of complement-fixing antigens. Purified soluble and particulate antigens comparable to those of other rickettsiae were obtained. In the present paper, the method of purification, physicochemical properties, and immunological specificity of the antigens are described.

MATERIALS AND METHODS

Rickettsial strains. (i) R. sennetsu. The Miyama strain, isolated in Fukuoka City by Misao and Kobayashi in 1953 (8), was used unless otherwise indicated. The Yoshimoto strain, isolated in Hiroshima Prefecture in 1958 (2), the Kojima strain, isolated in Kumamoto Prefecture in 1963 (9), and the Sugamori strain, isolated in Kitakyushu City in 1964 (9), were also used.

(ii) R. orientalis. The Gilliam strain (6) was used.

Animals. (i) Mice. Ten-week-old mice of the dd strain weighing 20 to 25 g, raised in the laboratory, were used.

(ii) Guinea pigs. Young guinea pigs weighing about 250 g were used.

Antiserum. (i) Antiserum to R. sennetsu. Immune guinea pig serum was prepared by the following method. Spleens of the infected mice were homogenized in phosphate-glutamate-sucrose buffer (PGS) (1) to make a 10% suspension and centrifuged at 1,000 rpm for 5 min. One milliliter of the supernatant was given to guinea pigs by intraperitoneal inoculation. Spleens of the infected animals were harvested 15 days after inoculation, and a 10% suspension in PGS was prepared as described above. One milliliter of the suspension was injected intraperitoneally into guinea pigs. On day 21 or 28, animals were bled out by heart puncture. Sera were

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separated and stored at −20 C. These serum samples were inactivated at 56 C for 30 min before use. It was confirmed in advance that such serum samples contained no antibody for normal control spleen antigens.

(ii) Antiserum to R. orientalis. Anti-Gilliam strain mouse immune serum was prepared as described previously (6, 7).

Complement-fixing antigens of R. orientalis. Soluble and particulate complement-fixing antigens of the Gilliam strain were prepared from infected yolk sac pools according to the method of Kobayashi et al. (5, 7, 11).

Complement fixation technique. The tests were performed by the procedures described previously (5, 6).

Physicochemical properties of the antigens. (i) Treatment of antigens by heat. Soluble and particulate antigens were heated in a water bath at 100 C for 30 min.

(ii) Ammonium sulfate precipitation of the soluble antigen. The soluble antigen, diluted to 10% with veronal-buffered saline solution, pH 7.3 (VBS) (6), was gradually added with 4 volumes of saturated ammonium sulfate (pH 7.3) at 4 C. It was then stirred at 4 C overnight. After centrifugation at 10,000 × g for 30 min, the supernatant was dialyzed in a distilled water for 24 h and then in VBS for 24 h at 4 C. The volume of the supernatant was adjusted to that of the original 100% antigen.

The sediment was washed with 80% saturated ammonium sulfate solution and dissolved in VBS. The solution was dialyzed in VBS for 24 h at 4 C and concentrated to the original volume of 100% antigen by ultrafiltration.

Preparation of spleen suspension from mice infected with R. sennetsu. Spleen suspension used as the source of rickettsia was prepared from mice infected with R. sennetsu and treated with cyclophosphamide (Endoan, Shionogi & Co. Ltd., Osaka).

The rickettsial inoculum was prepared from mouse spleens harvested 12 days after infection. Spleens were weighed and homogenized in a Waring blender with 9 volumes of PGS. The homogenate was centrifuged at 1,000 rpm for 5 min, and the supernatant was drawn off. A 0.2-ml amount of the suspension was inoculated into the mice intraperitoneally.

The method of drug administration described previously (14) was followed with a few modifications to facilitate the treatment of a large number of mice simultaneously. The drug was administered three times at 5-day intervals with a dose of 5.0 mg/mouse intraperitoneally. The first dose was given at the time of the rickettsial inoculation, and the second and the third doses were given 5 and 10 days after the rickettsial inoculation, respectively.

On days 12 to 14, when infected mice were severely sick, spleens were harvested, pooled, and weighed. A 10% spleen suspension in PGS was prepared by homogenization in a Waring blender and stored at −70 C until use. The weight of individual spleens averaged about 0.1 g.

Preparation of antigens. Basically, the first step for antigen purification was differential centrifugation followed by washing the rickettsia with a solution consisting of equal volumes of PGS and VBS. The second step was ether treatment to separate soluble and particulate antigens. The outlines of the procedure are presented in Fig. 1.

Frozen homogenate of infected spleens was thawed quickly in a water bath of 37 C, diluted in PGS to make a 5% suspension, and treated in a Waring blender for 3 min. The suspension was sedimented at 1,500 rpm for 10 min with a Tominaga 90 UV centrifuge with a horizontal rotor at 0 to 4 C. The supernatant was drawn off and centrifuged at 10,000 × g for 30 min with rotor no. 4. The sediment was suspended in an amount of 1:1 PGS-VBS to make a 5% suspension and homogenized in a Waring blender for 1 min. Then the homogenate was centrifuged at 10,000 × g for 30 min, and the sediment was resuspended in 1:1 PGS-VBS to make a 2.5% suspension.

After homogenization in a Waring blender for 1 min, 2 volumes of cold anesthetic ether (Sanraku-Ocean Co. Ltd., Tokyo) was added. The mixture was then shaken vigorously for 5 min and allowed to stand for 2 h at 4 C. This procedure was repeated three times, and then the mixture was allowed to stand overnight at 4 C. By this treatment there was clean separation into three layers: the upper ether layer, the thin middle layer, and the lower aqueous layer containing a small amount of precipitate.

The aqueous layer was separated and residual ether was evaporated under reduced pressure. After centrifugation of this aqueous layer at 10,000 × g for 30 min, the supernatant fluid containing the soluble antigen was concentrated to a volume of 1/20 by ultrafiltration with a Visking tube and dialyzed for 18 h in VBS. The final volume of the fluid was adjusted to the same weight as the original spleen by concentration with a collodion bag (Sartorius Co. Ltd., Göttingen), and it was used as the 100% soluble antigen.

The sediment was washed twice in VBS and then sedimented at 10,000 × g for 30 min. Finally, the precipitate was resuspended in VBS to obtain the weight of the original spleens and used as the 100% particulate antigen.

Both antigens were stored at −70 C until use. Normal mouse spleens were treated as described above to prepare the normal control antigens.

RESULTS

Medium for purification of antigens. During preliminary studies, PGS was used as the medium through the purification procedure. It gave unsatisfactory results in terms of removal of anti-complementary activity.

Thereafter, four medium samples were tested in the washing of rickettsiae and treatment with ether: PGS, VBS, and solutions of PGS and VBS at ratios of 2:1 and 1:1.

Titters of the soluble and particulate antigens treated in PGS were both 1:16, those treated in 2:1 PGS-VBS and 1:1 PGS-VBS were 1:32 for the soluble antigen and 1:8 for the particulate antigen, and those treated in VBS were 1:8 for the soluble antigen and 1:4 for the particulate antigen.
antigen. Anti-complementary activity was completely removed from antigens except those treated in PGS, which showed slightly higher antibody titers, probably due to nonspecific reactions (Fig. 2).

### Effect of heat treatment on antigens.
Titers of the soluble and particulate antigens before treatment were 1:64 and 1:32, respectively. After treatment, the soluble antigen showed no positive reaction, but the particulate antigen showed only a slight decrease. The results indicated that the soluble antigen was heat labile and the particulate antigen was heat stable (Fig. 3).

### Ammonium sulfate precipitation of the soluble antigen.
After treatment, the antigenic titer of the precipitate was 1:16, which was similar to that of the pretreated control antigen, whereas in the supernatant no antigenic activity was observed (Fig. 4).

**Cross-complement fixation test among the strains of R. sennetsu.** Antigenic characteristics of the soluble and particulate antigens of four strains of *R. sennetsu* (Miyayama, Yoshi-moto, Sugamori, and Kojima) were studied by cross-complement fixation tests. Very similar reaction patterns in both homologous and heterologous systems of the strains were observed in both the soluble and particulate antigens.

**Cross-complement fixation test between *R. sennetsu* and *R. orientalis.*** In the homologous system, titers of soluble and particulate antigens of the Miyayama strain were 1:32 and 1:16, respectively, and antibody titers of the immune serum against them were 1:64 and 1:128, respectively. Antigen titers of the Gilliam strain were 1:8 and 1:4, respectively, and antibody titers were 1:128 and 1:256, respectively. In the heterologous systems, positive reaction was observed in neither the Miyayama nor the Gilliam strain (Fig. 7).

**DISCUSSION**
Our previous report demonstrated that *R. sennetsu* propagates abundantly in spleens of

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**Fig. 1. Flow diagram of the procedures for purification of antigens.** The final volumes of antigens were adjusted to give the same weight as the original spleens.
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Fig. 2. Effect of medium samples used in the purification of antigens. Constituents of the medium samples examined are defined in the text. Original concentration of the antigens was 50%. Dilution of both antigen and antiserum is expressed as the reciprocal of the dilution. Black areas represent the positive reaction.

Fig. 3. Effect of heat treatment on soluble and particulate antigens. The treatment was performed by heating antigens at 100°C for 30 min. Black areas represent the positive reaction, and the open square represents the negative reaction. Original concentration of the antigens was 100%.

mice treated with cyclophosphamide (14). The mean infective titer of the rickettsia in spleens increased 100-fold or more over that of the non-drug-treated control mice. Since yolk sac tissue of chicken embryo, which was usually used as the source of antigens of other rickettsiae (5), was not available for R. sennetsu because of the low growth rate in the tissue, preparation of complement-fixing antigens was attempted by using a rickettsia-rich spleen suspension. Consequently, the soluble and particulate antigens
Several procedures have been reported for the method of purification of rickettsial antigens (5, 13). In this experiment, the method devised for purification of complement-fixing antigens of *R. orientalis* (7, 11) was followed with some modifications.

During preliminary studies, PGS was used as the medium to minimize harmful effects on the rickettsia during the purification procedure.

of the rickettsiae were purified with ether treatment.

For preparing the infected spleen suspension, the method of administration of cyclophosphamide was slightly modified in its timing and dose to facilitate treating many mice simultaneously: 5.0 mg/mouse, three times. It also gave good results in regard to the growth of rickettsiae in spleens.

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During preliminary studies, PGS was used as the medium to minimize harmful effects on the rickettsia during the purification procedure.
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Fig. 7. Cross-complement fixation test between *R. sennetsu* (Miyayama strain) and *R. orientalis* (Gilliam strain). Original concentration of the Miyayama strain was 100% and that of the Gilliam strain was 400%. Anti-Miyayama strain guinea pig serum was obtained 28 days after infection, and anti-Gilliam strain mouse serum was obtained 28 days after infection. Black areas represent the positive reaction and open squares represent the negative reaction.

However, it was found that traces of anti-complementary activity still remained in the antigens. Then PGS was replaced by VBS, and a washing procedure after differential centrifugation was interposed. Although anti-complementary activity was removed by using VBS, antigen titers were somewhat decreased.

Then solutions of PGS and VBS at different ratios were examined as the medium samples to increase efficiency of purification. Results indicated that the solution of 1:1 PGS-VBS was superior to others, since anti-complementary activity of the antigens purified in this medium was completely removed and the titer of the soluble antigen slightly elevated. Almost similar results were obtained by using 2:1 PGS-VBS.

Of the physicochemical properties of antigens, heat stability was examined. Results showed that the soluble antigen was heat labile and the particulate antigen was heat stable. In addition, the soluble antigen of *R. sennetsu* was precipitated by 80% saturation with ammonium sulfate without a decrease in the antigenic activity. These results suggest that a major constituent of the soluble antigen may be protein and that of the particulate antigen may be a non-protein substance. These characteristics were identical with those of *R. orientalis* (5, 11, 12). In contrast, the soluble antigens of *R. prowazeki* and *R. rickettsi* were heat stable and their particulate antigens were heat labile (5, 13).

Among four strains of *R. sennetsu* isolated in different districts of western Japan, there was no difference in antigenic characteristics as shown in cross-complement fixation tests using both the soluble and particulate antigens. The results were consistent with those obtained by immunofluorescent study (3). The immunological relationship between *R. sennetsu* and *R. orientalis* was also studied. Cross-reaction was not observed by complement fixation tests.

The data presented here provide a basis for further experiments on sennetsu rickettsiosis.

LITERATURE CITED

