Effect of Antiflagellar Serum in the Protection of Mice Against *Clostridium chauvoei*

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Specific antiflagellar serum of *Clostridium chauvoei* showed a powerful protective effect which prevented bacterial growth in the liver, but not in infected muscle, against intramuscular challenge with calcium chloride-activated spores in normal mice. No protective effect was observed in mice with polymorphonuclear leucocytes depleted by cyclophosphamide treatment. The antiflagellar serum had approximately the same protective effect in mice with macrophages blocked selectively by carrageenan as it did in normal mice. We suggest that the antiflagellar serum exerted its effect by opsonic function and that opsonized *C. chauvoei* was eliminated mainly by polymorphonuclear leucocytes rather than by macrophages.

Clostridium chauvoei is a sporeforming anaerobic bacterium which causes blackleg, a disease with economic impact, in cattle, sheep, and sometimes other ruminants. Vaccination for this disease is commonly done in many countries with formalinized whole culture (2). In contrast to other clostridial diseases caused by toxigenic organisms, in which toxoids serve as highly efficacious vaccines, we found earlier that the flagella of *C. chauvoei* are important for protective immunity in mice (Y. Tamura, N. Minamoto, and S. Tanaka, Microbiol. Immunol., in press). Moreover, the antiflagellar serum (anti-F serum) of this organism protects mice against challenge exposure. However, the mechanism by which antiflagellar immunization enhances survival in mice infected with *C. chauvoei* is still not fully understood.

Phagocytes are believed to contribute to protection against various bacteria as the final effector cells. For example, protection against *Listeria monocytogenes* (12), at least in the early stages, appears to depend mainly on macrophages, whereas *Pseudmonas aeruginosa* (12) and *Escherichia coli* (13) are killed effectively by polymorphonuclear leucocytes (PMN). To elucidate the protective mechanism of the flagella of *C. chauvoei*, the present study analyzed the involvement of cells in the protection of mice treated with specific anti-F serum, using mice that had been immunosuppressed by carrageenan (CG) or cyclophosphamide (CY).

MATERIALS AND METHODS

Animals. Outbred female ddy mice purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan, were used for the experiments when they were 6 weeks old. Rabbits of both sexes, all weighing about 3 kg, were obtained from a local breeder.

Bacterial strains. The Okinawa strain of *C. chauvoei* used in this study is used for vaccine production and for challenging mice in potency assays in Japan (1). The minimal lethal dose of this strain for mice inoculated by the intramuscular route was approximately 10 spores when they were inoculated with 3% calcium chloride solution. Nonflagellated mutant (NFM) FN-42 from the same strain was isolated by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and has been characterized previously (11).

Preparation of anti-F serum. The partially purified flagel-

lum preparation (PPF) was prepared from motile organisms of the Okinawa strain as described previously (Tamura et al., in press). Briefly, the organisms were grown anaerobically in a GasPak system (BBL Microbiology Laboratories, Cockeysville, Md.) in CLB broth (1) without glucose at 37°C until the late logarithmic phase, and then Formalin was added to a final concentration of 0.5%. The bacterial cells were suspended in 0.1 M Tris buffer (pH 8.0) at volumes of 1/40 of the original cultures. Flagella were sheared in a high-speed homogenizer (Hiscotron, Nichion Co., Ltd., Funabashi, Japan) at 15,000 rpm for 4 min. Deflagellated cells were removed by centrifugation at $6,500 \times g$ for 20 min, and the resulting supernatant was further centrifuged at $77,000 \times g$ for 90 min. The pellet was suspended in a small amount of 0.1 M Tris buffer and mixed with cesium chloride to a final density of 1.35 g/cm^3. After centrifugation at $73,000 \times g$ for 24 h, a band appearing at 1.33 g/cm^3 was harvest and dialyzed against 0.05 M Tris buffer (pH 7.2) and designated as PPF.

Rabbits were immunized by two subcutaneous injections (4 weeks apart) of 0.5 ml of PPF in 500 μg of protein emulsified in an equal amount of complete Freund adjuvant (Iatron Laboratories, Tokyo, Japan). At 13 days after the second injection, the rabbits were exsanguinated (anti-PPF serum). To prepare anti-F serum, anti-PPF serum was absorbed with the NFM by the following procedure to remove the O antibodies produced with the moiety derived from cell debris in PPF. Formalinized cells of strain FN-42 were washed three times with phosphate-buffered saline (pH 7.2) and were mixed with anti-PPF serum at 100 mg/ml. After incubation at 37°C for 2 h and then at 4°C for 18 h, the serum was separated by centrifugation. This procedure was repeated at least three times until the O antibodies disappeared. The resulting serum was sterilized by passage through a 0.22-μm filter and was stored at −20°C.

Tests for serological properties of anti-F serum. Details of the agglutination tests were described previously (Tamura et al., in press). H antigen from formalinized whole cells and O antigen from boiled cells were prepared from the Okinawa strain. The immobilization test was performed as follows. Sterilized tubes (10 by 1.2 cm) were placed in a 50°C water bath, and 2.7 ml of molten CLB broth with 0.15% agar was added to each tube. Twofold serial dilutions were made in phosphate-buffered saline. A volume of 0.3 ml of each serum dilution was gently mixed with soft agar. The Okinawa strain was grown anaerobically in heart infusion agar (Difco Labo-

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ratories, Detroit, Mich.) supplemented with 5% defibrinated sheep blood at 37°C for 24 h. Single colonies were stab-inoculated into each tube after the soft agar had cooled. The highest dilution of serum that inhibited the migration of the organism was termed the immobilization titer of the serum. The gel diffusion test was done by the method of Ouchterlony (9). PPF solubilized by treatment with 0.2% sodium dodecyl sulfate (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 60 min was used as the antigen. Values for the 50% effective dose (ED_{50}) of the serum in mice were measured as described previously (Tamura et al., in press).

Determination of bacterial growth in mice. Mice were given intramuscular inoculations in the middle of the right thigh; each inoculum consisted of 2.3×10^2 Okinawa-strain spores suspended in 0.25 ml of 3% calcium chloride solution. At various times after inoculation, groups of four mice were sacrificed to count the number of organisms in infected muscle and in the liver as indices of local and generalized infections, respectively. The whole muscle mass of an infected thigh and the liver were weighed and homogenized with nine volumes of anaerobic diluent A (8) (KH_2PO_4, 4.5 g; Na_2HPO_4, 6.0 g; L-cysteine monohydrochloride, 0.5 g; Tween 80, 0.5 g; agar, 1.0 g, in 1,000 ml of distilled water) with a glass homogenizer. Tenfold serial dilutions of the suspensions were made with the same diluent, and 0.05 ml of each dilution was spread on TF medium (Eiken Chemical Co., Ltd., Tokyo, Japan) supplemented with 5% defibrinated sheep blood. Colonies were counted after anaerobic culture at 37°C for 24 h.

Treatment of mice with anti-F serum. The anti-F serum was diluted 40-fold with phosphate-buffered saline, and 0.5 ml was injected intraperitoneally 3 h before challenge.

Treatment of mice with CG or CY. CG (Sigma, type II) was dissolved in distilled water and injected intraperitoneally (200 mg/kg) 24 h before challenge. CY (Sigma) was also dissolved in distilled water and injected intraperitoneally (150 mg/kg) 72 h before challenge.

Count of peripheral leukocytes. Blood specimens were collected by puncture of the retroorbital venous plexus. Total numbers of leukocytes were counted after staining with Türk solution, and differential counts were carried out after staining of the smears with Giemsa solution.

RESULTS

Serological properties of anti-F serum. Table 1 shows the serological properties of anti-F serum. The anti-H titer of anti-PPF serum employed was 163,840, whereas the anti-O titer was 640. As for absorption with NFM, anti-PPF serum completely eliminated O antibodies, as evidenced by significantly reduced O agglutinating titers. There was no difference in H titers between the two sera. These results were supported by gel diffusion analysis with solubilized PPF as the antigen (Fig. 1). Both sera gave a single common precipitation line corresponding to flagellin, but anti-PPF serum gave a different precipitation line as well. Although

TABLE 1. Serological properties of antiserum to the Okinawa strain

Anti-serum[a]	Agglutination titer		Immobiliza-tion titer	ED_{50} ($\times 10^{-3}$)[b]
	H	O		
PPF	163,840	640	1,280	5.3
Flagella	163,840	<20	1,280	6.3

[a] Antiserum was prepared as described in the test.
[b] ED_{50} values are indicated as the volume of serum in milliliters.
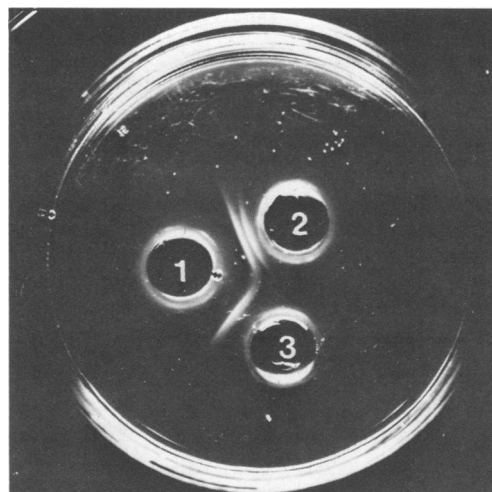

FIG. 1. Gel diffusion analysis of anti-PPF serum and anti-F serum to the solubilized PPF. Well 1, Solubilized PPF; well 2, anti-PPF serum; well 3, anti-F serum.

immobilization in soft agar also occurred at the same titers, the titers were approximately 100-fold lower than the corresponding titers of H agglutination. Bacterial growth was not entirely prevented in the CLB broth to which these sera were added to a final concentration of 10% (unpublished data). However, the ED_{50} values of these sera were high and were not significantly different.

Effect of anti-F serum on bacterial growth in muscle and liver. Mice pretreated with anti-F serum were protected completely from intramuscular challenge, although the mortality of untreated mice was 100% within 24 h (Fig. 2). Viable bacteria in infected muscle and liver were counted at various times after challenge. In the anti-F serum-treated mice, the numbers of bacteria in infected muscle increased by 48 h to reach over $10^7/g$ and then decreased progressively over 120 h. No bacteria were detected in the liver throughout the experiment. In the control mice, the numbers of bacteria increased markedly by 12 h to reach over $10^7/g$ in infected muscle and approximately $10^3/g$ in the liver. Thereafter, the organisms grew progressively in infected muscle and the liver until the mice died. In addition, bacterial growth in the normal serum-treated mice was similar to that in the control mice. Therefore, the elimination of *C. chauvoei* was apparent only in the presence of anti-F serum.

Effect of CG and CY on circulating leukocytes. To confirm the effect of CG and CY on the peripheral blood leukocytes of mice, the numbers of PMN, lymphocytes, and monocytes as macrophages were counted at 24 h after injection with CG and at 72 h after injection with CY (Fig. 3). Although the numbers of monocytes decreased to one-fourth of those in the untreated control after CG treatment ($P < 0.01$), that of PMN increased 1.7-fold. The numbers of PMN, on the other hand, decreased to one-eighth of those in the untreated controls after CY treatment ($P < 0.01$), and numbers of monocytes also decreased to one-fifth. The numbers of lymphocytes decreased to one-half of those in the controls after CG or CY treatment. In the phagocytes, these results indicated that CG mainly affected macrophages and CY affected both PMN and macrophages.

Effect of anti-F serum on bacterial growth in CG- and CY-treated mice. To determine the involvement of cells in protecting mice treated with anti-F serum, the mortality and
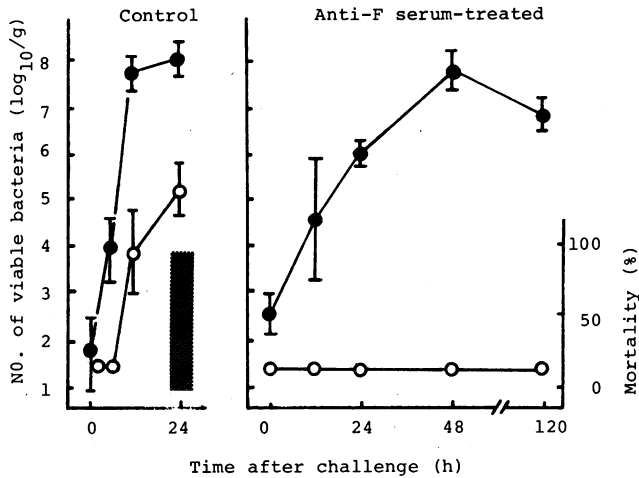
FIG. 2. Mortality and bacterial growth in mice treated with anti-F serum after intramuscular challenge. Symbols: ●, bacterial growth in infected muscle; ○, bacterial growth in the liver; ▓, cumulative mortality. Means of four mice ± standard errors are shown.

DISCUSSION

With *Vibrio cholerae*, the flagella appear to be necessary for the organism to attach itself to the intestinal mucosa to deliver its toxin efficiently (5, 7, 14). Immunization with crude flagellum vaccines decreases the number of radiolabeled vibrios attached to the mucosae of challenged rabbits (15). On the other hand, a recent report indicated that groups of mice immunized with *P. aeruginosa* flagellar antigen preparations showed higher survival rates when they were challenged locally in the burned area, and the protection appears to be due to immobilization of the microorganisms in the burned skin tissue (6). In contrast to these findings, little is known about the mechanism by which anti-flagellar immunization to mice against *C. chauvoei* offers protection against challenge, although the flagella are known to play an important role in protective immunity of this disease (Tamura et al., in press).

Our previous report suggested that the anti-flagellar serum plays an important role in passive protection (Tamura et al., in press). Our present results also support this suggestion. Evidence that passive protection might be due to anti-flagellar antibody was obtained by having the anti-PPF serum absorbed with NFM. The data showed no appreciable loss in ED_{50} between anti-PPF serum and anti-F serum (Table 1), but the anti-O titer of the serum was reduced below a detectable level by the absorption with NFM. With these considerations in mind, we attempted to elucidate this mechanism of anti-flagellar immunization on protection by the movement of challenged bacteria in mice treated with anti-F serum. Calcium chloride-activated spores injected intramuscularly without the presence of anti-F serum grew progressively in infected muscle and liver until the mice died. However, spores inoculated into anti-F serum-treated mice remained localized within the infected muscle throughout the experiment, and there was no systemic spread of bacteria (Fig. 2). Thus, the numbers of viable bacteria increased markedly in infected muscle of anti-F serum-

bacterial growth in infected muscle and liver after intramuscular challenge were compared among CG-treated, CY-treated, and untreated control mice pretreated with anti-F serum (Fig. 4). All CG-treated mice survived throughout the experiment, whereas 75% of the CY-treated mice died within 48 h. The numbers of viable bacteria in CG-treated mice were essentially the same as those in untreated control mice with anti-F serum (Fig. 2). No bacteria were detected in the liver at any period during the experiment. However, progressive growth of challenged bacteria in the liver was observed in Cy-treated mice. The numbers of viable bacteria increased rapidly by 24 h to reach over 10^5/g, although the numbers in infected muscle were similar to those in untreated controls.
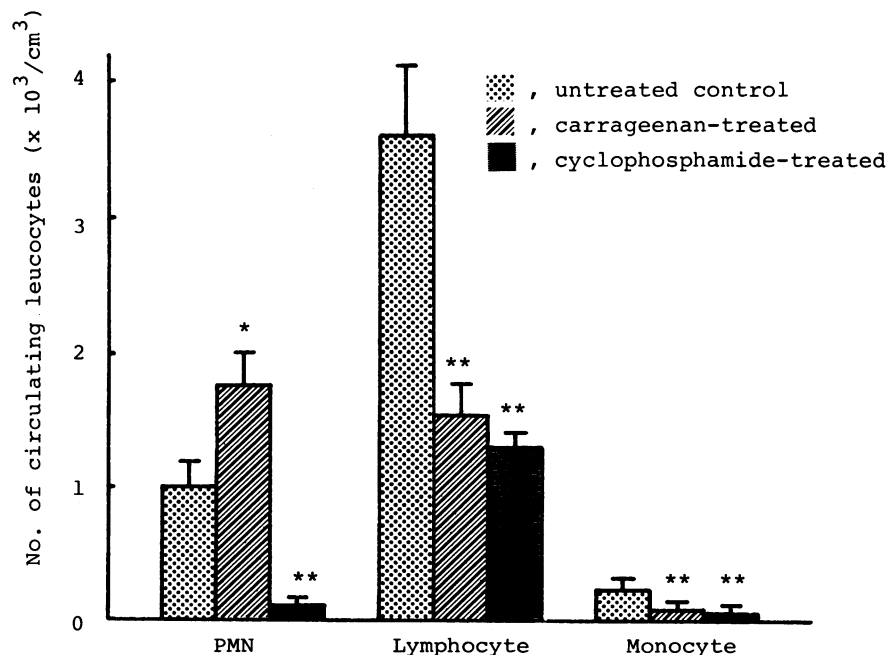

FIG. 3. Effects of CG and CY on the number of circulating leukocytes in the peripheral blood of mice. Significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) compared with untreated controls are shown. Means of 10 mice ± standard errors are shown.

Carrageenan-treated   Cyclophosphamide-treated
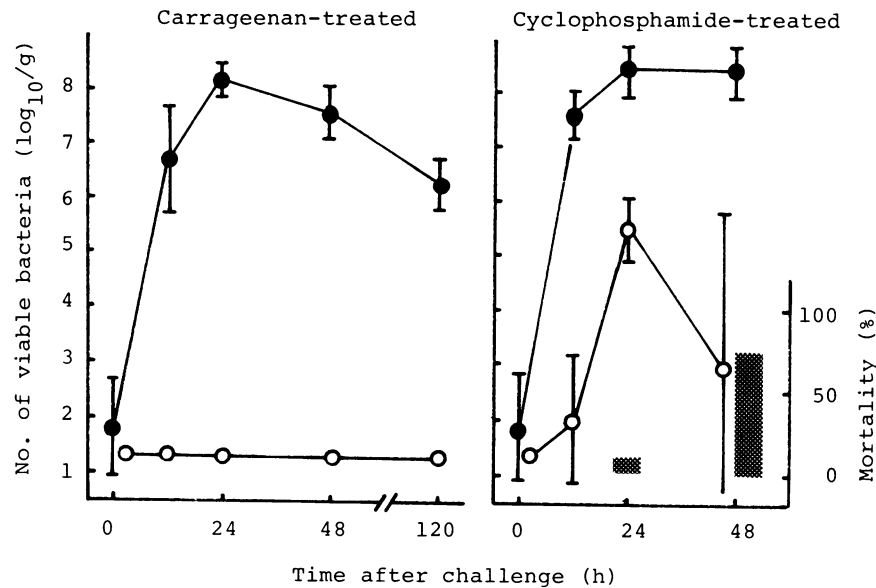

Time after challenge (h)

FIG. 4. Mortality and bacterial growth in mice treated with CG and anti-F serum, and CY and anti-F serum, after intramuscular challenge. Symbols: ●, bacterial growth in infected muscle; ○, bacterial growth in the liver; ▓, cumulative mortality. Means of four mice ± standard errors are shown.

treated mice, as in control mice, after challenge. In general, mice display a natural resistance to *C. chauvoei* challenge without calcium chloride. Calcium chloride has been used in experimental infections for many years (1, 10). The activating effect of calcium chloride, when injected along with spores of *C. chauvoei*, has been attributed to localized tissue damage, which produces an ideal focus for the germination of spores and multiplication of vegetative cells under anaerobic condition (10). In the control mice inoculated with calcium chloride-activated spores, bacterial growth was detected in infected muscle, but not in the liver until 6 h. Thereafter, the organisms spread into systemic organs as growth was detected in the liver (Fig. 2). Therefore, calcium chloride may act as an activator in the pathogenesis of *C. chauvoei* infection, particularly in local infection. Perhaps the protective mechanism involves prevention of the systemic spread of bacteria by the anti-F serum.

In such an early stage of infection, phagocytes such as PMN or macrophages have a more important role in the elimination of the challenged bacteria than immunologically reactive cells do (12, 13). CG selectively damages cells of the macrophage series, but it does not damage PMN (12). On the other hand, it is well known that CY depletes PMN and macrophages as well as immune lymphocytes (3, 4). Our experimental systems were essentially similar to those used in these reports (Fig. 3). Therefore, we tried to establish which phagocytes make the main contribution to protection against *C. chauvoei*. CY dramatically increased the mortality rate of mice treated with anti-F serum after challenge, whereas CG did not affect it at all (Fig. 4). Thus, protection against this organism depends mainly on PMN but not on macrophages, and the protective effects of anti-F serum are exhibited only in the presence of PMN. This interpretation was supported by the pattern of bacterial growth in the liver as an index of generalized infection. Although no bacteria were detected in CG-treated mice given anti-F serum, progressive bacterial growth was observed in CY-treated mice, even in the presence of anti-F serum. We therefore suggest that anti-F serum exerts its effect by opsonic function and

that opsonized *C. chauvoei* is eliminated mainly by PMN rather than by macrophages. Further studies on role of PMN in the protection of mice are necessary to prove this speculation.

LITERATURE CITED

1. Azechi, H., K. Seto, Y. Mukawa, and K. Suzuki. 1962. Studies on the potency test of blackleg vaccine. Jpn. J. Vet. Sci. 24:267–275. (In Japanese with English summary.)
2. Bruner, D. W., and J. H. Gillespie. 1966. *Clostridium chauvoei*, p. 361–367. *In* Hagan's infectious diseases of domestic animals. Cornell University Press, Ithaca, N.Y.
3. Buhles, W. C., Jr., and M. Shifrine. 1977. Adjuvant protection against bacterial infection in granulocytopenic mice. J. Infect. Dis. 136:90–95.
4. Buhles, W. C., Jr., and M. Shifrine. 1977. Effects of cyclophosphamide on macrophages, functions and progenitor cells. RES J. Reticuloendothel. Soc. 21:285–297.
5. Guentzel, M. N., and L. J. Berry. 1975. Motility as a virulence factor for *Vibrio cholerae*. Infect. Immun. 11:890–897.
6. Holder, I. A., R. Wheeler, and T. C. Montie. 1982. Flagellar preparations from *Pseudomonas aeruginosa*: animal protection studies. Infect. Immun. 35:276–280.
7. Jones, G. W., and R. Freter. 1976. Adhesive properties of *Vibrio cholerae*: nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect. Immun. 14:240–245.
8. Ogawa, K., H. Yokota, M. Kimura, and T. Mitsuoka. 1981. Effects of administration of *Bacillus subtilis* strain BN on intestinal flora of weanling piglets. Jpn. J. Vet. Sci. 43:771–775.
9. Ouchterlony, O. 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. 26:507–515.
10. Princewill, T. J. T. 1965. Effect of calcium chloride on germination and pathogenicity of spores of *Clostridium chauvoei*. J. Comp. Pathol. 75:343–351.
11. Tamura, Y., N. Minamoto, and S. Tanaka. 1982. Isolation and characterization of non-flagellated and paralyzed flagellated mutants of *Clostridium chauvoei*. Jpn. J. Vet. Sci. 45:417–420.
12. Tatsukawa, K., M. Mitsuyama, K. Takeya, and K. Nomoto.

1979. Differing contribution of polymorphonuclear cells and macrophages to protection of mice against *Listeria monocytogenes* and *Pseudomonas aeruginosa*. J. Gen. Microbiol. 115:161–166.

13. Tsuru, S., K. Nomoto, M. Mitsuyama, Y. Zinnaka, and K. Takeya. 1981. Importance of polymorphonuclear leucocytes in protection of mice against *Escherichia coli*. J. Gen. Microbiol.

122:335–338.

14. Yancey, R. J., D. L. Willis, and L. J. Berry. 1978. Rose of motility in experimental cholera in adult rabbits. Infect. Immun. 22:387–392.

15. Yancey, R. J., D. L. Willis, and L. J. Berry. 1979. Flagella-induced immunity against experimental cholera in adult rabbits. Infect. Immun. 25:220–228.