INFECTION AND IMMUNITY, Nov. 1983, p. 618–622
0019-9567/83/110618-05$02.00/0

Vol. 42, No. 2

Stimulation of Complement Production in Mice by N^{α}-(*N*-Acetylmuramyl-L-Alanyl-D-Isoglutamine)-N^{ε}-Stearoyl-L-Lysine

NOBUKO ENDO,[1] TOSHIRO OKUDA,[1] YASUAKI OSADA,[2]* AND HIROSHI ZEN-YOJI[1]

Department of Microbiology and Immunology, School of Health Science, Kyorin University, Tokyo 192,[1] and Research Institute, Daiichi Seiyaku Co., Ltd., Tokyo 134, Japan[2]

Received 18 April 1983/Accepted 3 August 1983

N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutamine)-N^{ε}-stearoyl-L-lysine, a synthetic muramyl dipeptide analog, stimulated the production of the third component of complement (C3) in mice. The serum concentration of C3 was elevated significantly by subcutaneous treatment with a single dose (10 to 100 μg per mouse) of the adjuvant 24 h before assay of the serum. Thereafter, the concentration decreased gradually with time and returned to the normal level on day 4 to 5. Immunoelectrophoretic analysis of the serum revealed that the decrease in serum C3 could not be accounted for by the cleavage to C3a and C3b. By intermittent treatment with the adjuvant on every fifth day, a significant increase in serum C3 was repeated. However, no continuous retention of the serum level of C3 was established even during continuous treatment with the adjuvant once a day for 10 consecutive days. Instead, in this case, the level of C3 increased repeatedly at almost 5-day intervals.

It is well known that each of the components of complement plays an important role in primary host defense mechanisms against microbial infections via alternative pathways even if an individual has no specific antibody (11, 17, 28, 33). Actually, a host deficient in serum complement with a hard inflammatory response or with undernourishment has been found to be susceptible to repeated infections (1, 3, 5, 27).

Muramyl dipeptide (MDP) and some of its analogs (MDPs) have been reported to enhance the nonspecific resistance of animals to microbial infections (6, 14, 15, 22, 23, 25). Osada et al. have recently found that polymorphonuclear leukocytes (PMNs) were activated in vitro and in vivo by an MDP analog, N^{α}-(*N*-acetyl-muramyl-L-alanyl-D-isoglutamine)-N^{ε}-stearoyl-L-lysine [MDP-Lys(L18)] (24). We supposed that, in addition to PMN activation, increase in serum concentration of C3 may in part contribute to the augmentation of nonspecific resistance to microbial infections in mice treated with MDP-Lys(L18). Thus, this paper deals with the stimulative effect of MDP-Lys(L18) on the complement productivity in mice.

MATERIALS AND METHODS

MDP. MDP-Lys(L18) (Daiichi Seiyaku Co., Ltd., Tokyo, Japan) was dissolved in Dulbecco phosphate-buffered saline (PBS, pH 7.4) (Nissui Seiyaku Co., Ltd., Tokyo, Japan) at a concentration of 500 μg/ml just before use and diluted with PBS to between 1×10^{-1} and 7×10^{-1}. This lot of MDP-Lys(L18) was verified to be free of lipopolysaccharide by the *Limulus* lysate assay, using *Limulus* Test Wako (Wako Pure Chemical Industries, Tokyo, Japan).

Animals. Outbred young male adult (4 weeks of age) mice of strain STD:ddY, weighing 20 to 25 g (Shizuoka Cooperative for Experimental Animals, Hamamatsu, Japan), were placed in quarantine for 1 week to check for infectious disorders and then used for the experiments. Drinking water and food were given ad libitum.

Treatment and sampling. Groups of five mice were injected subcutaneously (s.c.) with 0.2 ml of each dilution of MDP-Lys(L18) according to the following schedules: (i) a single dose of 10, 40, 70, or 100 μg per mouse on day 0, (ii) intermittent dosing with 10 μg per mouse at 2-, 3-, and 5-day intervals, and (iii) a dose of 10 μg per mouse once a day for 10 consecutive days. As controls, groups of five mice injected s.c. with 0.2 ml of PBS were used. In experiment I, the mice treated with the adjuvant or with PBS were anesthetized with ether after 24 h after treatment, and blood samples were collected by heart puncture. From other groups of mice with 10 μg of the adjuvant per mouse, the blood samples were gathered every day from 1 to 6 days after treatment. In experiment II, the blood sampling was carried out every day for mice treated at 2- and 3-day intervals and on days 0, 4, 5, 6, 9, 10, 11, 14, 15, and 16 for mice treated at 5-day intervals, respectively. In experiment III, the sampling was carried out every day 24 h after the last treatment. In these experiments, blood was collected from separate groups of mice on each occasion. After incubation of the blood samples in an ice bath for 1 h, the sera were

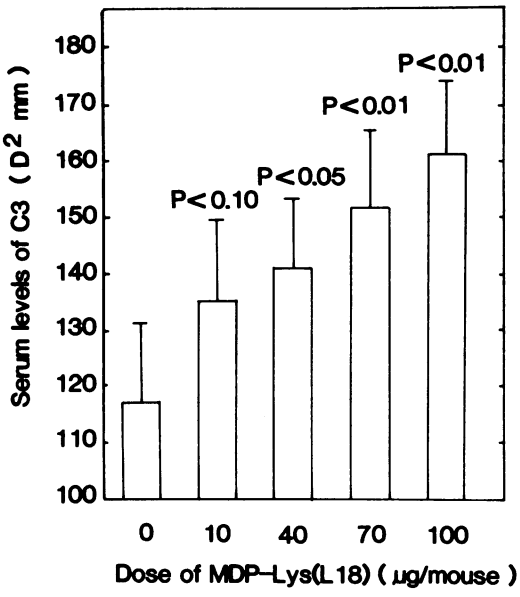

FIG. 1. Dose-dependent increase in serum C3 level in mice with MDP-Lys(L18). Groups of five mice were treated s.c. with 10, 40, 70, or 100 μg of MDP-Lys(L18) per mouse or with PBS, and blood samples were collected from them by heart puncture 24 h later. The sera prepared by the procedures described in the text were applied to the SRID assay for measurement of C3 protein. The results are expressed as the mean ± standard deviation. The level of significance compared with the values of mice with PBS, which are indicated as 0, was assessed by Student's *t* test.

prepared by centrifugation at 3,000 rpm for 10 min at 2°C and preserved in a deep freezer at −80°C until needed for use.

Measurement of C3. The serum concentrations of C3 were measured by a modification of the method of Mancini et al. (16), the single radial immunodiffusion (SRID) method, using an agarose plate containing anti-mouse C3 rabbit serum. The plates were prepared by mixing 2% agarose (Hoechst Japan Ltd., Tokyo, Japan) with the same volume of 5% anti-mouse C3 rabbit serum (Fuji Zoki Pharmaceutical Co., Ltd., Tokyo, Japan) diluted with 0.01 M EDTA-Veronal buffer, pH 8.6, at 55°C, and pouring the mixture into glass slits containing U-frames (80 by 80 by 0.1 cm). After the gelation of agarose, the upper glass plate was removed carefully, and holes 2 mm in diameter were made in the agarose layer. A total of 16 holes were made on each agarose plate, and 4-μl quantities of each serum sample were poured into the holes with a microsyringe. After incubation of the plates at room temperature for 48 h, the diameter of the precipitated zone was measured with a zone reader (magnification, ×7), and its square was taken as the amount of C3 in serum.

Immunoelectrophoresis of serum. In vitro and in vivo activation of serum C3 by the adjuvant was examined by immunoelectrophoresis (IEP). In the in vitro experiment, sera from untreated mice were supplemented with MDP-Lys(L18) to give concentrations of 4 to 250 μg/ml and were incubated at 37°C for 30 or 60 min. In vivo activation was tested by using the sera derived from the mice treated with 10 μg of the adjuvant per mouse. The control samples were the sera of the untreated mice (negative control) and those incubated in vitro with 5 mg per ml of zymosan (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 30 or 60 min (positive control). The IEP plates were prepared by pouring 1% agarose dissolved in 0.01 M EDTA-Veronal buffer, pH 8.6, onto glass plates (11 by 9 cm). After the agarose had solidified horizontally, holes 2 mm in diameter were made in it and filled with the serum. Ninety minutes after the start of electrophoresis at 40 mA, 20-μl quantities of anti-mouse C3 serum were poured into the slits (2 mm in width) made in the agarose, and the plates were incubated overnight at room temperature in a humidified box.

Significance test. The results of each experiment were expressed as the mean ± standard deviation, and statistical analysis were performed by Student's *t* test. The test was made on multiple ($n = 5$) observations in a single experiment.

RESULTS

Dose-dependent increase in serum C3. Groups of five mice were injected s.c. with 0.2 ml of PBS or of the adjuvant solution in PBS at a concentration of 50, 200, 350, or 500 μg/ml (equivalent to 10, 40, 70, or 100 μg per mouse), and samples of serum derived from each group of mice were tested 24 h after treatment. Figure 1 shows the

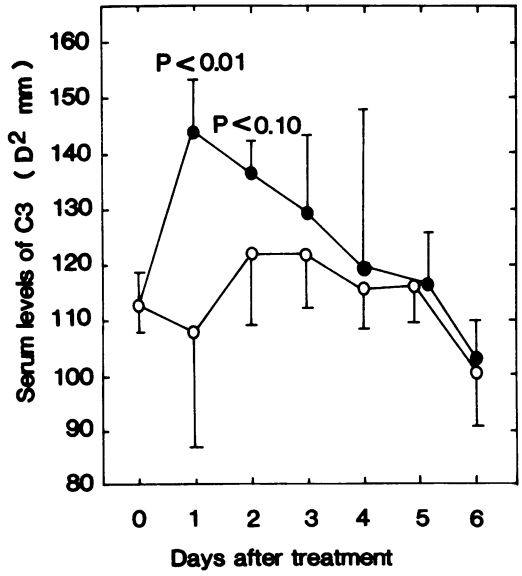

FIG. 2. Retention of the serum C3 level after treatment with MDP-Lys(L18). Groups of five mice were treated s.c. with 10 μg of MDP-Lys(L18) per mouse (●) or with PBS (○) on day 0, and the sera were prepared from separate groups of mice every 24 h after treatment for 6 consecutive days. The amount of C3 in serum was measured by the SRID assay described in the text. The results were expressed as the mean ± standard deviation, and their significance was tested by Student's *t* test.

increases in serum level of C3 with increase in dose of the adjuvant. The increases of C3 in mice injected with 10, 40, 70, and 100 μg of the adjuvant per mouse were 16.4, 22.4, 31.2, and 39.6%, respectively, and were statistically significant at the levels of 10, 5, and 1%.

Retention of serum level of C3. After treatment of each mouse with 10 μg of MDP-Lys(L18), the serum levels of C3 in separate groups of mice were measured once a day for 6 consecutive days. Figure 2 shows that the level was maximum ($P < 0.01$) 1 day after treatment (day 1) and then gradually decreased with time, and on day 4 to 5 returned to the normal level. Therefore, mice were treated with the adjuvant at 5-day intervals. The result was a retention pattern for serum C3 similar to that seen with a single dose (Fig. 3). At each treatment, the levels increased significantly ($P < 0.01$) 1 day after treatment, except for day 6, and returned to normal 4 days later. In an attempt to keep the level high, treatment was made continuously once a day for 10 consecutive days or intermittently at 2- or 3-day intervals. However, the increased level on day 1 decreased with time irrespective of any

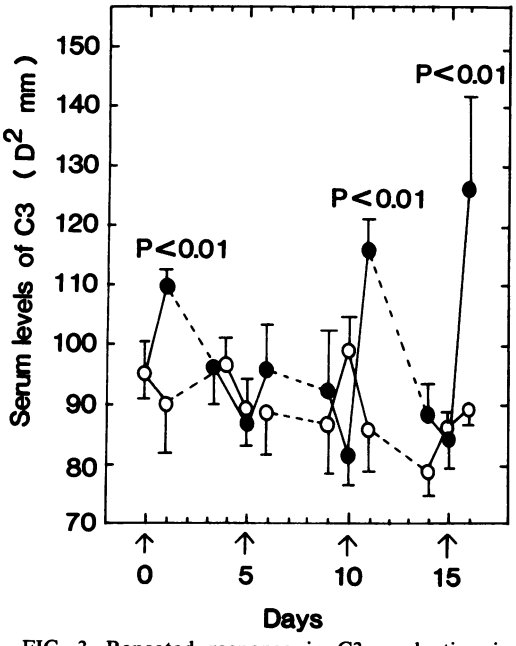

FIG. 3. Repeated response in C3 production in mice by intermittent treatment with MDP-Lys(L18). Groups of five mice were treated s.c. with 10 μg of MDP-Lys(L18) per mouse (●) or with PBS (○) at 5-day intervals, and the blood samples were collected from separate groups of mice on days 0, 1, 4, 5, 6, 9, 10, 11, 14, 15, and 16. The amount of C3 in serum was measured by the SRID assay described in the text. The results were expressed as the mean ± standard deviation, and their significance was tested by Student's *t* test.

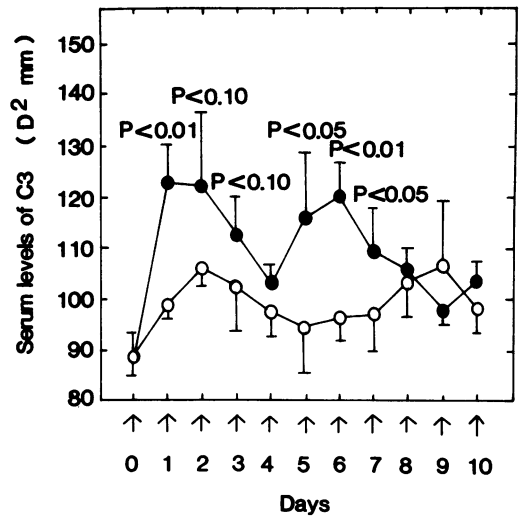

FIG. 4. Intermittent response of C3 production in mice continuously treated with MDP-Lys(L18). Groups of five mice were treated s.c. with 10 μg of MDP-Lys(L18) per mouse (●) or with PBS (○) once a day for 10 consecutive days, and blood was collected from separate groups of mice every day. The amount of C3 in the serum was measured by the SRID assay. The results were expressed as the mean ± standard deviation, and their significance was tested by Student's *t* test.

subsequent treatment and returned to the normal level on day 4 (Fig. 4). Almost the same pattern was repeated once, and the level rose again somewhat on day 6. The intermittent treatment at 2- or 3-day intervals also failed to keep the serum level of C3 (data not shown).

IEP of sera. To determine C3 activation by MDP-Lys(L18), we applied the samples of serum derived from mice treated with 10 μg of the adjuvant and those incubated in vitro with the adjuvant to the IEP test. Figure 5 shows the IEP pattern of C3 in serum derived from the mice treated with the adjuvant. The band of C3 protein activated by zymosan (positive control) changed from β_{1C}-globulin to β_{1A}-globulin, whereas proteins in serum derived from mice with the adjuvant did not. In vitro incubation of serum with the adjuvant also resulted in no change in the IEP pattern of C3 (data not shown). These results suggest that C3 protein was not activated either directly or indirectly by MDP-Lys(L18).

DISCUSSION

It is well known that C3 or C3-like proteins such as cobra venom factor are found in both lower and higher vertebrates and play an important role in nonspecific and specific host defense (1–3, 8, 9, 14, 19, 20, 26). We supposed that the

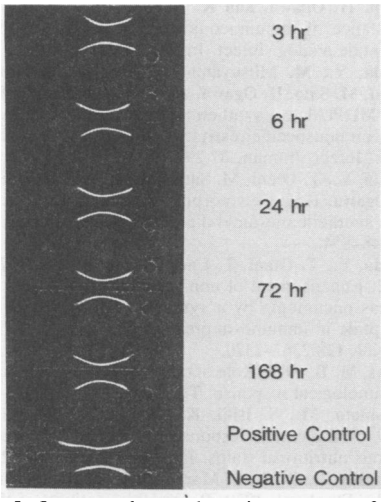

FIG. 5. Immunoelectrophoretic pattern of C3 in sera of mice treated with MDP-Lys(L18). The sera derived from mice treated with 10 μg of MDP-Lys(L18) per mouse at each time indicated in the figure were applied to the IEP assay described in the text. As control, the serum from untreated mice (negative control) and that incubated in vitro with zymosam (positive control) were used. The bands of C3 protein in sera of mice with PBS and those from mice with the adjuvant were illustrated on the upper and lower side of each agarose slit, respectively.

complement systems might be involved in the resistance mechanisms of animals to microbial infections, which are stimulated by MDPs (6, 14, 15, 22, 23, 25). To clarify its involvement in the enhancement of defense by MDP-Lys(L18) against microbial infections in mice, a quantitative and qualitative examination was made of serum C3 in mice treated with or without the adjuvant. As a result, the serum concentration of C3 increased with increased doses of the adjuvant. The level reached the maximum 24 h after treatment and then decreased with time irrespective of any subsequent treatment. On day 4 to 5, the level returned to normal.

It has been reported that MDPs have activating effects on macrophages (12, 18, 29, 30, 32). Most of the components of complement are synthesized and released from the monocyte-macrophage system cells (4, 7, 13, 21). By the IEP analysis, however, little cleavage (activation) of C3 to C3a and C3b by the adjuvant was found. This fact suggests that the increase in serum concentration of C3 is hardly due to the amplification of C3 production by macrophages subsequent to the consumption (cleavage) of C3. On the basis of these findings, we feel confident that the elevation of the serum concentration of C3 by treatment with MDP-Lys(L18) is attributable to stimulation by the adjuvant of C3 biosynthesis by the effector cells. Actually, we found that MDP-Lys(L18) stimulated C3 generation by cultured mouse macrophages in vitro (N. Endo, T. Okuda, Y. Osada, and H. Zen-Yoji, manuscript in preparation).

To be exact, we don't know how the increased serum C3 functions in the nonspecific resistance to microbial infections. It may contribute in part to opsonization of the microbial invader, which facilitates the phagocytosis of microorganisms by PMNs and macrophages activated by the adjuvant (12, 24, 25, 29). It may be that the components of complement in serum and in the body fluids resulting from the activity of the adjuvant may contribute to effective activation of the PMN functions when the activation of the alternative pathway of complement is triggered by any of stimulant, including microorganisms (10, 28, 31). The finding that PMN activation by the adjuvant was greater in vivo than in vitro (24) may support this supposition.

The cause of the periodic increase in serum C3 in mice treated continuously with the adjuvant is obscure. The second to fourth treatment seemed to be ineffective in maintaining a high level of C3 in the serum. To make clear the cause, further in vitro studies must be made of the stimulative effect of the adjuvant on the generation of C3 by macrophages and its mechanisms. Studies of functional correlations between effector cells, PMNs, macrophages, and lymphoid cells and studies of the physiological disposition of this adjuvant are also required.

LITERATURE CITED

1. **Alper, C. A., N. Abramson, R. B. Johnston. J. H. Jandl, and F. S. Rosen.** 1970. Increased susceptibility to infection associated with abnormalities of complement-mediated functions and of the third component of complement (C3). N. Engl. J. Med. **282**:349–354.
2. **Alper, C. A., and D. Balavitch.** 1976. Cobra venom factor: evidence for its being altered cobra C3 (the third component of complement). Science **191**:1275–1276.
3. **Alper, C. A., H. R. Colten, F. S. Rosen, A. R. Rabson, G. M. Macnab, and J. S. S. Gear.** 1972. Homozygous deficiency of C3 in a patient with repeated infections. Lancet **ii**:1179–1181.
4. **Bentley, C., D. Bitter-Suerman, U. Hadding, and V. Brade.** 1976. In vitro synthesis of factor B of the alternative pathway of complement activation by mouse peritoneal macrophages. Eur. J. Immunol. **6**:393–398.
5. **Chandra, R. K.** 1975. Serum complement and immunoconglutinin in malnutrition. Arch. Dis. Child. **50**:225–229.
6. **Chedid, L., M. Parant, F. Parant, P. Lefrancier, J. Choay, and E. Lederer.** 1977. Enhancement of nonspecific immunity to *Klebsiella pneumoniae* infection by a synthetic immunoadjuvant (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) and several analogs. Proc. Natl. Acad. Sci. U.S.A. **74**:2089–2093.
7. **Colten, H. R.** 1976. Biosynthesis of complement. Adv. Immunol. **22**:81–95.
8. **Day, N. K. B., H. Gewurz, R. Johannsen, J. Finstad, and R. A. Good.** 1970. Complement and complement-like activity in lower vertebrates and invertebrates. J. Exp. Med. **132**:941–950.
9. **Dukor, P., G. Schuman, R. H. Gisler, M. Dierich, W. König, U. Hadding, and D. Bitter-Suermann.** 1974. Complement-dependent B-cell activation by cobra venom factor and other mitogens. J. Exp. Med. **139**:337–345.

10. **Fearon, D. T., S. Ruddy, P. H. Schur, and W. R. McCabe.** 1975. Activation of the properdin pathway of complement in patients with gram-negative bacteria. N. Engl. J. Med. **292**:937–940.
11. **Hirsch, R. L.** 1982. The complement system: its importance in the host response to viral infection. Microbiol. Rev. **46**:71–85.
12. **Imai, K., and A. Tanaka.** 1981. Effect of muramyl dipeptide, a synthetic bacterial adjuvant, on enzyme release from cultured mouse macrophages. Microbiol. Immunol. **25**:51–62.
13. **Kawamoto, Y., M. Ueda, H. Ichikawa, and A. Miyama.** 1979. Complement proteins and macrophages. I. Quantitative estimation of factor B produced by mouse peritoneal macrophages. Microbiol. Immunol. **23**:987–995.
14. **Kiersenbaum, F., and R. W. Ferraresi.** 1979. Enhancement of host resistance against *Trypanosoma cruzi* infection by the immunoregulatory agent muramyl dipeptide. Infect. Immun. **25**:273–278.
15. **Krahenbuhl, J. L., S. D. Sharma, R. W. Ferraresi, and J. S. Remington.** 1981. Effects of muramyl dipeptide treatment on resistance to infection with *Toxoplasma gondii* in mice. Infect. Immun. **31**:716–722.
16. **Mancini, G., A. O. Carbonara, and J. F. Hermans.** 1965. Immunochemical quantitation of antigen by single radial immunodiffusion. Immunochemistry **2**:235–254.
17. **Müller-Eberhard, H. J., and R. D. Schrisber.** 1980. Molecular biology and chemistry of the alternative pathway of complement. Adv. Immunol. **29**:32–34.
18. **Nagao, S., A. Tanaka, Y. Yamamoto, T. Koga, K. Onoue, T. Shiba, K. Kusumoto, and S. Kotani.** 1979. Inhibition of macrophage migration by muramyl peptides. Infect. Immun. **24**:308–312.
19. **Naoguchi, J.** 1979. The phylogenesis and otogenesis of the complement system. Jpn. J. Clin. Med. **37**:930–936. (In Japanese.)
20. **Nishioka, K., K. Kawamura, T. Kawashima, K. Shimada, and M. Kogure.** 1976. C3; junction of humoral and cellular factors. Excerpta Med. Int. Congr. Ser. **414**:230–235.
21. **Ooi, Y. E., D. E. Harris, P. J. Edelson, and H. R. Colten.** 1980. Post-translational control of complement (C5) production by resident stimulated mouse macrophages. J. Immunol. **124**:2077–2081.
22. **Osada, Y., M. Mitsuyama, K. Matsumoto, T. Une, T. Otani, H. Ogawa, and K. Nomoto.** 1982. Stimulation of resistance of immunocompromised mice by a muramyl dipeptide analog. Infect. Immun. **37**:1285–1288.
23. **Osada, Y., M. Mitsuyama, T. Une, K. Matsumoto, T. Otani, M. Sato, H. Ogawa, and K. Nomoto.** 1982. Effect of L18-MDP(Ala), a synthetic derivative of muramyl dipeptide, on nonspecific resistance of mice to microbial infections. Infect. Immun. **37**:292–300.
24. **Osada, Y., T. Otani, M. Sato, T. Une, K. Matsumoto, and H. Ogawa.** 1982. Polymorphonuclear leukocyte activation by a synthetic muramyl dipeptide analog. Infect. Immun. **38**:848–854.
25. **Osada, Y., T. Otani, T. Une, H. Ogawa, and K. Nomoto.** 1982. Enhancement of non-specific resistance to pseudomonas pneumonia by a synthetic derivative of muramyl dipeptide in immunosuppressed guinea pigs. J. Gen. Microbiol. **128**:2361–2370.
26. **Pepys, M. B.** 1976. Role of complement in the induction of immunological response. Transplant. Rev. **32**:93–120.
27. **Sakamoto, M., S. Ishii, K. Nishioka, and K. Shimada.** 1979. Complement response after bacterial infection in various nutritional states. Immunology **38**:421–427.
28. **Schreiber, R. D., D. C. Morrison, E. R. Podack, and H. J. Müller-Eberhard.** 1979. Bactericidal activity of the alternative complement pathway generated from 11 isolated plasma proteins. J. Exp. Med. **149**:870–882.
29. **Tanaka, A., S. Nagao, K. Imai, and R. Mori.** 1980. Macrophage activation by muramyl dipeptide as measured by macrophage spreading and attachment. Microbiol. Immunol. **24**:547–557.
30. **Taniyama, T., and T. Holden.** 1979. Direct augmentation of cytolytic activity of tumor-derived macrophage and macrophage cell lines by muramyl dipeptides. Cell Immunol. **48**:369–374.
31. **Taylor, P. W.** 1983. Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. Microbiol. Rev. **47**:62–70.
32. **Whal, S. M., L. M. Whal, J. B. McCarthy, L. Chedid, and S. E. Margenhagen.** 1979. Macrophage activation by mycobacterial water soluble components and synthetic muramyl dipeptide. J. Immunol. **122**:2226–2231.
33. **Winkelstein, J. A., M. R. Smith, and H. S. Shin.** 1975. The role of C3 as an opsonin in the early stages of infection. Proc. Soc. Exp. Biol. Med. **149**:397–401.