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Unimpaired Function of Human Phagocytes in the Presence of Phagocytosis-Resistant Group A Streptococci

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The resistance to phagocytosis of the group A streptococci has been attributed mainly to the presence of the surface antigen, M protein. In the present study, we addressed the question of whether the phagocytosis resistance of the group A streptococci is due to their abilty to impair the function of the phagocytic cells. The results of these studies demonstrate that the presence of a large excess of a phagocytosis-resistant strain of streptococci does not significantly interfere with either the antibody-independent or the antibody-dependent phagocytosis of streptococci. Apparently, a phagocytosis-resistant strain of streptococci does not bring about a generalized deactivation of the phagocytic plasma membrane. This suggests that if the resistance of the group A streptococci is due to any deactivating influence at all on the phagocytic plasma membrane, it is likely to be confined to the contact area of the cocci with the phagocyte.

It is widely recognized that the resistance to phagocytosis of the group A streptococci by human granulocytes is mainly due to the presence of M protein on their cell surface (9). Hence, M protein is considered, by far, a major virulence factor for these bacteria. Antibodies to the M protein neutralize its antiphagocytic property and facilitate phagocytosis of the bacteria. More than 75 serotypes of the streptococcal M protein have been recognized over the years, and the neutralizing activity of the M antibody is essentially type specific.

The mechanism by which the M protein impedes the phagocytosis of the group A streptococci is not yet fully understood. Some recent studies have shown that M protein is associated with qualitative defects in opsonization by complement (4, 7, 12) and that it interferes with the receptor binding of the deposited complement components. (17). Other studies have suggested that fibrinogen inhibits the complement-mediated opsonization of the streptococci by binding to the surface M protein (18, 19). Structural studies have demonstrated that M protein, which has an alpha-helical coiled-coil structure similar to mammalian coiled-coil proteins, extends as long, flexible fibrils from the cell surface of the bacterium (10, 13, 15). The N-terminal domain of the M protein, which is distal to the bacterial cell surface (5, 13), has a significantly high negative charge, which in turn may play a role in the biological function of the M molecule (11).

In the present study, we addressed the question of whether the phagocytosis resistance of the group A streptococci is due to their ability to impair the function of the phagocytes. We therefore examined whether the presence of a phagocytosis-resistant strain of streptococci would have any influence on (i) the phagocytosis of a streptococcal strain that is otherwise susceptible to phagocytosis (antibody-independent phagocytosis) and/or (ii) the phagocytosis of a resistant strain rendered susceptible to phagocytosis due to opsonization by its antibody (antibody-dependent phagocytosis).

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MATERIALS AND METHODS

Streptococcal strains. *Streptococcus pyogenes* strains B788 (type 5), D471 (type 6), and C126/59/7 (type 43) were from the Rockefeller University collection. Strains B788 (type 5) and D471 (type 6) are resistant to phagocytosis by human granulocytes and were routinely enriched for the respective M proteins by rotation in normal human blood (3). Strain C126/59/7 (type 43) has previously been demonstrated to be a phagocytosis-susceptible strain (6, 20).

Mit-C treatment of type 5 streptococci. A 50-ml log-phase culture of the type 5 streptococci (strain B788) grown in Todd-Hewitt broth was sedimented by centrifugation, washed once with Todd-Hewitt broth, and resuspended in 5 ml of the same. A sample was removed for bacterial counts, and the remainder of the suspension was treated with mitomycin C (Mit-C; 50 μg/ml, final concentration; Schwarz Mann, Orangeburg, N.J.) for 1 h at 37°C, in the dark and with rotation at 16 rpm. The cells were pelleted by centrifugation, washed several times in sterile phosphate-buffered saline (PBS) (pH 7.4) to remove unreacted Mit-C, and resuspended to the original volume with PBS. Samples were removed to check for viability of cells by the pour-plate method, and the remainder of the suspension was stored at −70°C until used. Examination of the pour plates indicated the absence of viable cells after Mit-C treatment. The number of CFU in the Mit-C-treated type 5 streptococcal suspension was therefore based on the counts determined before the Mit-C treatment of the cocci.

Bactericidal assay. The bactericidal assay was carried out essentially as described by Lancefield (8). Assays were carried out in fresh, heparinized whole blood from nonimmune human donors. Samples (100 μl) of the type 43 streptococcal suspension were mixed with 100 μl of a suspension of Mit-C-treated type 5 streptococci, 100 μl of PBS, and 400 μl of heparinized human blood, and the mixture was incubated at 37°C for 3 h. To maximize contact between streptococci and phagocytes, the mixture was rotated end over end at 16 rpm during the course of incubation. Nonrotated controls of the reaction mixtures were run in parallel to determine the influence of the presence of Mit-C-treated type 5 streptococci on the proliferation of type 43

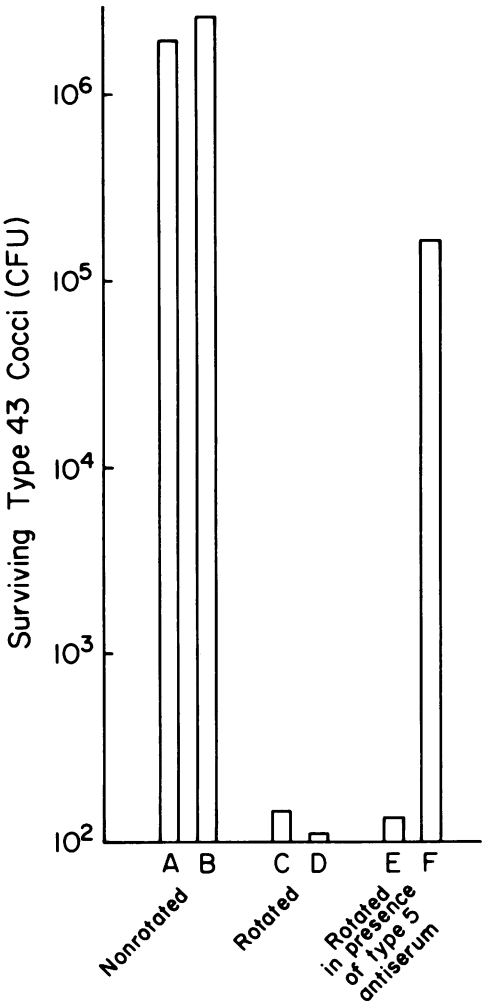

FIG. 1. Influence of Mit-C-treated type 5 streptococci on the phagocytosis of type 43 streptococci. Type 43 cocci inoculum, 5.49 $\times$ 10^4 CFU. Mit-C-treated type 5 cocci were present at 3.8 $\times$ 10^7 CFU. CFU presented on the histogram represent the counts from a 100-μl sample of the assay mixture at the end of the assay period. Nonrotated, conditions unfavorable for phagocytosis; rotated, conditions favorable for phagocytosis. Bars A, C, and E, Type 43 cocci in the absence of Mit-C-treated type 5 cocci. Bars B, D, and F, Type 43 cocci in the presence of Mit-C-treated type 5 cocci. The type 5 cocci were unopsonized in bars B and D, but were opsonized in bar F. Type 5 antiserum, but no type 5 cocci, were present in bar E.

streptococci under nonphagocytizing conditions. To ensure sufficient competition by the resistant strain, the Mit C-treated type 5 streptococci were used at an approximately 1,000-fold excess over the susceptible strain. Based on the average neutrophil count in normal human blood of 3.8 $\times$ 10^6/ml (1), the ratio of the CFU of the resistant strain to neutrophils in the assay system was of the order of 25:1. Thus, the ratio of the resistant type 5 CFU to neutrophils to susceptible cocci CFU was of the order of 1,000:40:1. Since the average chain length of the type 5 cocci was about six cocci, the ratio in terms of single cocci to neutrophils was of the order of 150:1. Control experiments were also run in which the type 5 cocci were opsonized. In these tests, 100 μl of the type 5 antiserum was used in place of PBS. At the end of the 3-h incubation period, the number of surviving organisms was determined by dilution of the assay mixture with sterile PBS, and plating 100-μl samples of the appropriate dilution by the pour-plate method in the presence of sheep blood.

In tests aimed at examining the influence of the presence of the resistant type 5 streptococci on antibody-mediated phagocytosis, experiments similar to those described above for the type 43 streptococci were carried out with type 6 streptococci in the presence or absence of 100 μl of type 6 antiserum.

Antisera. Antisera to heat-killed suspensions of type 5 and type 6 bacteria were prepared in rabbits as previously described (14).

RESULTS

Phagocytosis of susceptible type 43 streptococci in the presence of resistant type 5 streptococci. To determine the influence of a resistant strain of streptococci on the phagocytosis of a susceptible strain of streptococci, we carried out a bactericidal assay of the susceptible type 43 strain in the absence and in the presence of the resistant type 5 streptococci. To follow the phagocytosis of the susceptible strain in the presence of the resistant cocci without interference due to proliferation of the latter, a live preparation of the susceptible strain and a nonproliferating preparation of the resistant strain were used in the test system. To minimize the alteration of the bacterial surface structures, the resistant strain was rendered nonproliferative by treatment with Mit-C, a DNA-intercalating agent (16).

When the type 43 cocci were incubated without rotation in human blood (nonrotated conditions) they proliferated (Fig. 1, bar A). The degree of this proliferation was essentially unaltered in the presence of an approximately 700-fold excess of Mit-C-treated type 5 streptococci (bar B). However, when the type 43 cocci were rotated in the presence of human blood, they were readily ingested and killed (bar C). When a similar rotation was carried out in the presence of Mit-C-treated type 5 streptococci (bar D), the type 43 cocci were ingested and killed essentially to the same degree as in the absence of the type 5 streptococci (bar C). Thus, it is apparent that the presence of a nearly 700-fold excess of the phagocytosis-resistant type 5 streptococci did not interfere with either the growth or the phagocytosis of the susceptible type 43 cocci.

Although the type 5 streptococci by themselves resist phagocytosis, type-specific antibodies are capable of opsonizing these bacteria and thus facilitate their phagocytosis. Therefore, to ensure that the neutrophils were indeed present at limiting concentrations and that the assay system permitted the measurement of the influence, if any, of the resistant type 5 cocci on the function of the neutrophils, control experiments were run in which the type 5 cocci were opsonized. The type 5 antiserum by itself had no significant influence on the phagocytosis of the type 43 cocci (Fig. 1, bar E). The colony counts were essentially the same as in the absence of the type 5 antiserum (Fig. 1, bars C and E). However, the phagocytosis of the type 43 cocci were significantly inhibited in the presence of a large excess of the opsonized type 5 cocci (bar F). These results clearly indicate that under the present experimental conditions, the concentration of the neutrophils is indeed limiting.

Phagocytosis of opsonized type 6 streptococci in the presence of the resistant type 5 streptococci. Like type 5 streptococci, type 6 streptococci by themselves are resistant to ingestion by human granulocytes, and opsonization of these cocci by type-specific antiserum facilitates their phagocytosis. To determine whether the presence of a phagocytosis-resistant streptococcus has any influence on antibody-mediated

phagocytosis of a second streptococcus, the phagocytosis of opsonized type 6 streptococci was studied in the presence of Mit-C-treated type 5 streptococci. Like the type 43 cocci under nonrotated conditions, proliferation of the type 6 cocci was unaffected in the presence of Mit-C-treated type 5 streptococci (Fig. 2, lanes A and B). Similarly, neither the resistance to phagocytosis of unopsonized type 6 cocci (Fig. 2, lanes C and D) nor the susceptibility of the opsonized type 6 cocci was influenced significantly by the presence of Mit-C-treated type 5 cocci (Fig. 2, lanes E and F). Thus, it is apparent that Mit-C-treated type 5 streptococci did not significantly interfere with the antibody-mediated phagocytosis of the type 6 streptococci.

DISCUSSION

Earlier studies have demonstrated that the resistance to ingestion of the group A streptococci does not depend on the cocci being in an actively dividing state. It was observed that streptococci retain their ability to resist ingestion by phagocytes after they have been killed by heat, mercury-arc irradiation, or several chemical agents (21). In those studies, the susceptibility of cocci to ingestion was scored on the basis of the percentage of neutrophils containing the streptococci, i.e., on a morphological basis. In other studies, Beachey and Stollerman (2) have demonstrated that the phagocytes are not morphologically affected in the presence of live phagocytosis-resistant streptococci.

In the present study, we examined the susceptibility of streptococci to ingestion in a bactericidal assay system, which allows for scoring of the surviving cocci. The results clearly demonstrate that the presence of a nearly 700- to 1,000-fold excess of the phagocytosis-resistant type 5 cocci does not significantly interfere either with the phagocytosis of the susceptible type 43 streptococci, i.e., antibody-independent phagocytosis, or with the phagocytosis of opsonized type 6 streptococci, i.e., antibody-dependent phagocytosis. If the resistant streptococci brought about generalized deactivation of the phagocytic plasma membrane, then ingestion of the susceptible type 43 cocci, as well as that of the opsonized type 6 cocci, should be inhibited in the presence of the resistant cocci. If, on the other hand, the influence of the resistant cocci on the phagocytes is confined to the segment of the plasma membrane at or around the contact site of the resistant cocci, then ingestion of the susceptible cocci should be unaltered. The results of the present study demonstrate that even at a ratio of resistant streptococci to phagocytes of 150:1, there is no significant influence on either the antibody-dependent or the antibody-independent phagocytosis. It is therefore clear that the presence of the resistant cocci does not bring about a generalized impairment of the phagocytic function. Thus, it is apparent that if the resistance of the group A streptococci to phagocytosis, which is correlated with the presence of the surface M antigen, is due to a deactivating influence on the phagocytes, it is likely to be a local effect at or around the contact site of the resistant cocci with the phagocytic plasma membrane.

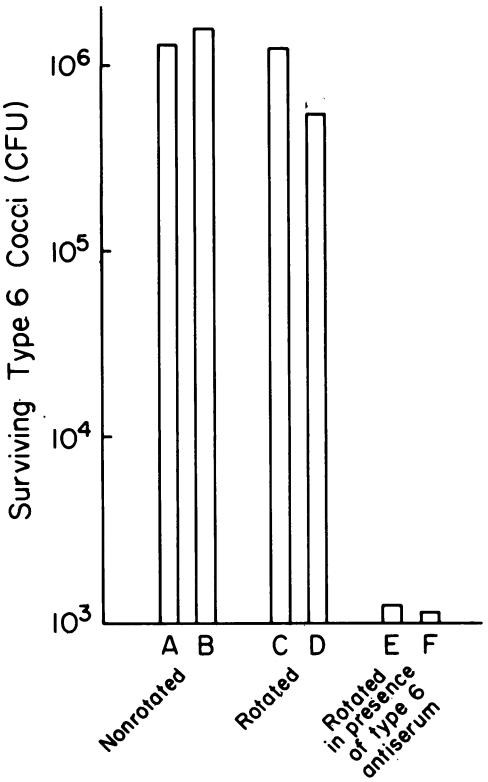

FIG. 2. Influence of Mit-C-treated type 5 cocci on the phagocytosis of opsonized type 6 streptococci. Type 6 cocci inoculum, 1.77×10^4 CFU. Mit-C-treated type 5 cocci were present at 3.8×10^7 CFU. Nonrotated and rotated refer to the experimental conditions that are unfavorable and favorable for phagocytosis, respectively. Bars A, C, and E, Type 6 cocci in the absence of Mit-C-treated type 5 cocci. Bars B, D, and F, Type 6 cocci in the presence of Mit-C-treated type 5 cocci. Type 6 cocci were unopsonized in bars C and D, but were opsonized in bars E and F.

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LITERATURE CITED

1. **Altman, P. L.** 1961. Biological handbook, p. 126. *In* D. S. Dittmer (ed.), Blood and other body fluids. Federation of the American Society for Experimental Biology, Washington, D.C.
2. **Beachey, E. H., and G. H. Stollerman.** 1971. Toxic effects of streptococcal M protein on platelets and polymorphonuclear leukocytes in human blood. J. Exp. Med. **134**:351–365.
3. **Becker, C. G.** 1964. Selection of group A streptococci rich in M protein from populations poor in M protein. Am. J. Pathol. **44**:51–60.
4. **Bisno, A. L.** 1979. Alternate complement pathway activation by group A streptococci: role of M protein. Infect. Immun. **26**:1172–1176.
5. **Fischetti, V. A., K. F. Jones, B. N. Manjula, and J. R. Scott.** 1984. Streptococcal M6 protein expressed in *Escherichia coli*. Localization, purification, and comparison with streptococcal-derived M protein. J. Exp. Med. **159**:1083–1095.
6. **Hosein, B., and V. A. Fischetti.** 1979. Binding and stimulation of opsonic antibodies by group A streptococci lacking anti-phagocytic activity, p. 19. *In* M. T. Parker (ed.), Pathogenic streptococci. Reedbooks Ltd., Surrey, England.

7. **Jacks-Weis, J., Y. Kim, and P. P. Cleary.** 1982. Restricted deposition of C3 on M^+ group A streptococci: correlation with resistance to phagocytosis. J. Immunol. **128:**1897–1902.
8. **Lancefield, R. C.** 1959. Persistence of type-specific antibodies in man following infection with group A streptococci. J. Exp. Med. **110:**271–292.
9. **Lancefield, R. C.** 1962. Current knowledge of type-specific M antigens of group A streptococci. J. Immunol. **89:**307–313.
10. **Manjula, B. N., and V. A. Fischetti.** 1980. Tropomyosin-like seven residue periodicity in three immunologically distinct streptococcal M proteins and its implications for the anti-phagocytic property of the molecule. J. Exp. Med. **151:**695–708.
11. **Manjula, B. N., B. Trus, and V. A. Fischetti.** 1985. Presence of two distinct regions of the coiled-coil structure of the streptococcal Pep M5 protein: relationship to mammalian coiled-coil proteins and implications to its biological properties. Proc. Natl. Acad. Sci. USA **82:**1064–1068.
12. **Peterson, P. K., D. Schmeling, P. P. Cleary, B. J. Wilkinson, Y. Kim, and P. G. Quie.** 1979. Inhibition of alternate complement pathway opsonization by group A streptococcal M protein. J. Infect. Dis. **139:**575–585.
13. **Phillips, G. N., Jr., P. F. Flicker, C. Cohen, B. N. Manjula, and V. A. Fischetti.** 1981. Streptococcal M protein: alpha-helical coiled-coil structure and arrangement on the cell surface. Proc. Natl. Acad. Sci. USA **78:**4689–4693.
14. **Rotta, J., R. M. Krause, R. C. Lancefield, W. Everly, and H. Lackland.** 1971. New approaches for the laboratory recognition of M types of group A streptococci. J. Exp. Med. **134:** 1298–1315.
15. **Swanson, J., K. C. Hsu, and E. C. Gotschlich.** 1969. Electron microscopic studies on streptococci. I. M antigen. J. Exp. Med. **130:**1063–1091.
16. **Szybalski, W., and V. N. Iyer.** 1967. The mitomycins and porfiromycins, p. 211–245. *In* D. Gottlieb and P. D. Shaw (ed.), Antibiotics. I. Mechanism of action. Springer-Verlag, New York.
17. **Weis, J. J., S. K. Law, R. P. Levine, and P. P. Cleary.** 1985. Resistance to phagocytosis by group A streptococci: failure of deposited complement opsonins to interact with cellular receptors. J. Immunol. **134:**500–505.
18. **Whitnack, E., and E. H. Beachey.** 1982. Antiopsonic activity of fibrinogen bound to M protein on the surface of group A streptococci. J. Clin. Invest. **69:**1042–1045.
19. **Whitnack, E., J. B. Dale, and E. H. Beachey.** 1984. Common protective antigens of group A streptococcal M proteins masked by fibrinogen. J. Exp. Med. **159:**1201–1212.
20. **Wiley, G. G., and P. N. Bruno.** 1972. The M antigens: variations and inter-relationships, p. 235–249. *In* L. W. Wannamaker and J. M. Matsen (ed.), Streptococci and streptococcal diseases. Academic Press, Inc., New York.
21. **Wiley, M. D., and A. T. Wilson.** 1956. The ability of group A streptococci killed by heat or mercury irradiation to resist ingestion by phagocytes. J. Exp. Med. **103:**15–36.