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Influence of Serum Concentration on Opsonization by the Classical and Alternative Complement Pathways

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In this investigation of bacterial opsonization by the serum complement system, the importance of using various serum concentrations and of performing kinetic studies is demonstrated.

Optimal phagocytosis of most bacterial species requires an effective opsonic source including complement or immunoglobulin. Opsonically active C3b molecules, covalently bound to the bacterial surface to promote adherence to phagocytes, can be formed from C3 by direct activation of the alternative complement pathway or by immunoglobulin activation of the classical complement pathway. The literature is replete with what appears to be conflicting data regarding the specific complement pathway responsible for opsonization of certain bacterial species or strains in dilute serum. Several groups of investigators (4-6, 10, 11,14) have shown that the classical complement pathway is required for optimal opsonization of *Staphylococcus aureus*, *Streptococcus pneumoniae*, group B streptococci, *Escherichia coli*, and *Pseudomonas aeruginosa*. However, others (1, 3, 4, 6, 7, 9, 11) have observed that these bacteria, in addition to several other species, are primarily opsonized via the alternative complement pathway. These apparent discrepancies may be related to a heterogeneity of opsonic requirements among strains (6, 12) or to differences in methodology. Many investigators have not independently studied the processes of opsonization and phagocytosis, few have performed kinetic studies, and most have examined opsonization by using serum concentrations of 10% or less.

Although strains of *S. aureus* and *S. pneumoniae* can be effectively opsonized via the alternative complement pathway, kinetic studies clearly indicate that an intact classical complement pathway is required for optimal opsonization (5, 12, 13). Verbrugh and co-workers (12) recently confirmed this observation and also reported that dilution of normal serum primarily affects the function of the classical complement pathway by decreasing concentrations of early complement components. These findings may elucidate some of the differences in opsonic requirements among strains as previously described. It is possible that opsonization of most bacterial species is mediated via the classical complement pathway at high serum concentrations and that as serum is diluted below a critical concentration of early complement components, classical complement pathway activity may be significantly diminished, resulting in opsonization by the alternative complement pathway.

To test the hypothesis that the serum concentration determines which complement pathway is most effectively utilized, we investigated opsonization of *S. aureus* 502A, *S. pneumoniae* XXIII, and *E. coli* ON2 in various concentrations of normal, C2-deficient, and heat-inactivated sera.

Normal human serum from six donors was pooled and stored at −70°C until used. Heat-inactivated serum was prepared by heating thawed serum at 56°C for 30 min. Serum from a patient with a genetically determined complete and selective absence of C2 (C2-deficient serum) was used to study opsonization in the absence of an intact classical complement pathway. Pure suspensions of normal human polymorphonuclear leukoctyes (PMN) were used in all experiments. Hank's balanced salt solution containing 0.1% gelatin was used for serum dilutions and PMN suspension. Opsonization and phagocytosis assays measuring PMN uptake of [^{3}H]thymidine-labeled bacteria were performed as described previously (10); the ratio of bacteria to PMN was about 10:1.

As shown in Fig. 1, *S. aureus* 502A was rapidly and effectively opsonized in 100 and 10% normal serum, with opsonization being complete by 1 min in 100% serum and by 5 min in 10% serum. In contrast, opsonization in 1% normal serum proceeded significantly more slowly, requiring 60 min for completion, and there was no opsonic activity in 0.1% serum (<10% PMN uptake). Heat-inactivated normal serum contained about one-third to one-half the opsonic activity of normal serum. Although undiluted C2-deficient serum was an effective opsonic source after incubation for 15 min, the rate of opsonization was

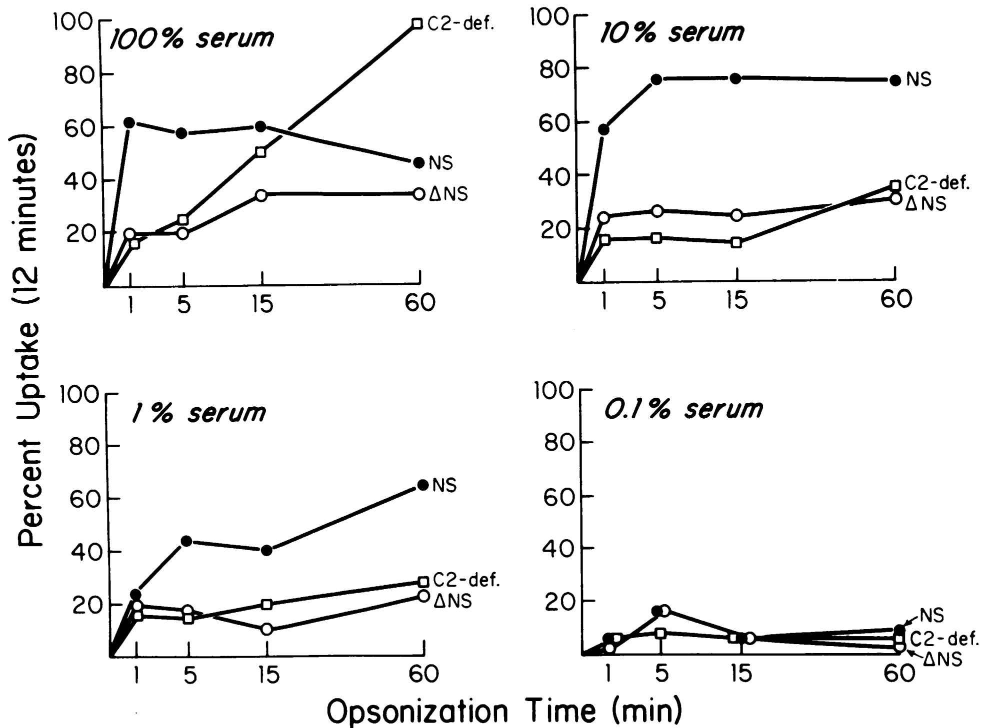

FIG. 1. *Phagocytosis of S. aureus 502A by PMN after incubation in human serum. Bacteria were opsonized for 1, 5, 15, and 60 min in 100, 10, 1, and 0.1% concentrations, respectively, of normal serum (●), heat-inactivated normal serum (○), and C2-deficient serum (□) before presentation to PMN. Phagocytosis was quantitated by measuring leukocyte-associated radioactivity at 12 min.*

significantly slower when compared with that of normal serum. At concentrations of 10 and 1%, C2-deficient serum was a poor opsonin.

Figure 2 shows that *S. pneumoniae* XXIII could be rapidly opsonized in 100% normal serum and that opsonization proceeded at progressively slower rates as serum was diluted to concentrations of 50 and 25%. There was no heat-labile opsonic activity in 10% normal serum and no heat-stable activity at any serum concentration (<10% uptake). C2-deficient serum was as effective as normal serum at all concentrations when opsonization was permitted to proceed for 60 min. However, kinetic studies demonstrate that the rate of opsonization in C2-deficient serum was significantly slower when compared with that of normal serum.

Figure 3 displays the kinetics of opsonization of *E. coli* ON2. Bacteria incubated in 100 and 50% concentrations of normal serum were fully opsonized by 1 min, whereas incubation in 25, 10, and 1% normal serum required 5, 15, and 60 min, respectively, for completion of opsonization. One percent serum was minimally opsonic (17% uptake, data not shown). Heat-inactivated serum was a poor opsonic source at all concentrations (<10% uptake). C2-deficient serum was a less effective opsonin than normal serum, and as previously shown with *S. aureus* 502A and *S. pneumoniae* XXIII, the rate of opsonization was significantly slower when compared with that of normal serum.

The results of these kinetic studies demonstrate that although opsonization via the alternative complement pathway occurs, an intact classical complement pathway is required for optimal opsonization of *S. aureus* 502A, *S. pneumoniae* XXIII, and *E. coli* ON2. The importance of kinetic studies when examining the opsonic activity of various sera and bacteria should be emphasized. For example, we might have concluded that opsonization of *S. pneumoniae* XXIII and *E. coli* ON2 occurred via the alternative complement pathway had we studied opsonization only after 60 min of incubation. Also, the serum concentration is critical when bacterial opsonization is studied. In this investigation, all three species were optimally opsonized in undiluted serum via activation of the classical complement pathway since opsonization in normal serum was complete within 1 min in contrast to C2-deficient serum, which had no opsonic

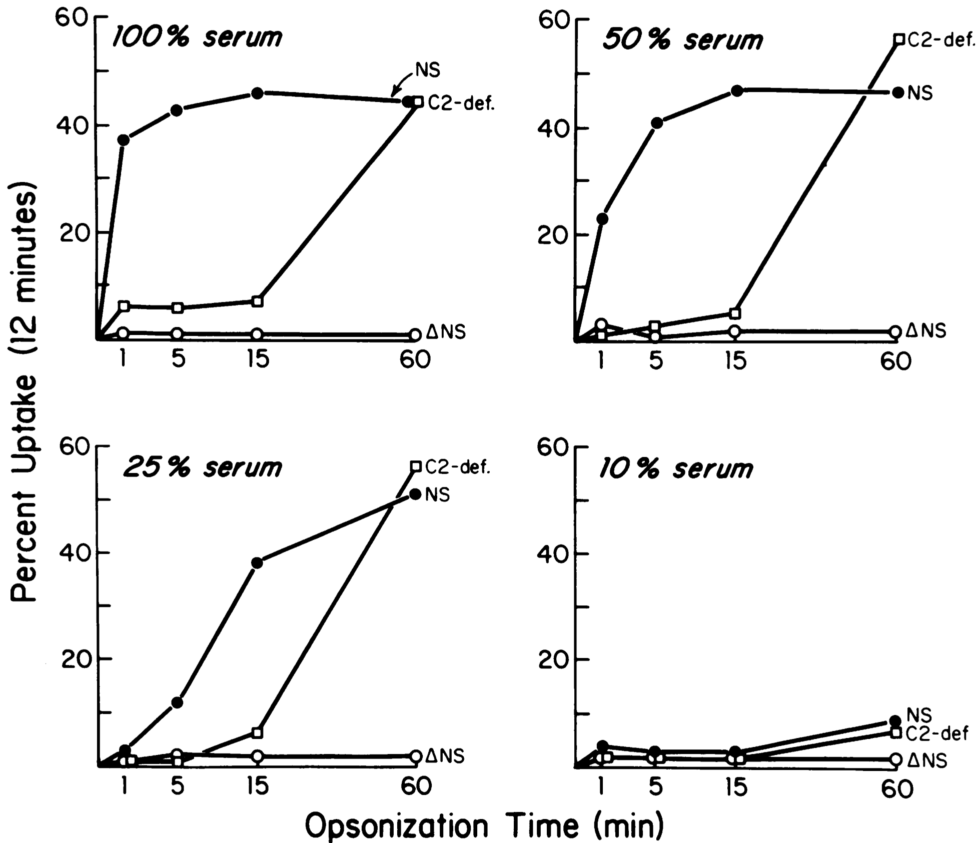

FIG. 2. *Phagocytosis of S. pneumoniae XXIII by PMN after incubation in human serum. Bacteria were opsonized for 1, 5, 15, and 60 min in 100, 50, 25, and 10% concentrations, respectively, of normal serum (●), heat-inactivated normal serum (○), and C2-deficient serum (□) before presentation to PMN. Phagocytosis was quantitated by measuring leukocyte-associated radioactivity at 12 min.*

activity after 1 min of incubation. The kinetics of opsonization of dilute normal serum and undiluted C2-deficient serum are similar, suggesting that opsonization in dilute serum is mediated by the alternative complement pathway. When normal human serum is diluted, a critical concentration occurs for each species in which the early-reacting complement components are reduced to suboptimal amounts, resulting in opsonization which proceeds via the alternative complement pathway. The serum concentrations below which opsonization is mediated primarily via the alternative complement pathway appear to be between 10 and 1% for *S. aureus* 502A, 50 and 25% for *S. pneumoniae* XXIII, and 25 and 10% for *E. coli* ON2. It is possible that dilution of immunoglobulin contributes to the observed reduction in classical complement pathway activity in dilute serum. However, recent data from Verbrugh et al. (12) support the hypothesis that dilution of the early-reacting complement pathway components rather than immunoglobulin accounts for the loss in classical complement pathway activity in relatively dilute serum.

An interesting phenomenon observed at high concentrations of normal serum with *E. coli* ON2 is that of significantly less phagocytosis of bacteria opsonized for 60 min as compared with those opsonized for 1 and 5 min. This finding remains unexplained. However, it is possible that the decrease in opsonization observed with increasing opsonization periods in undiluted serum results from the proteolytic cleavage of C3b from the bacterial surface by C3b-inactivator and the cofactor βIH (8). This possibility is currently under investigation.

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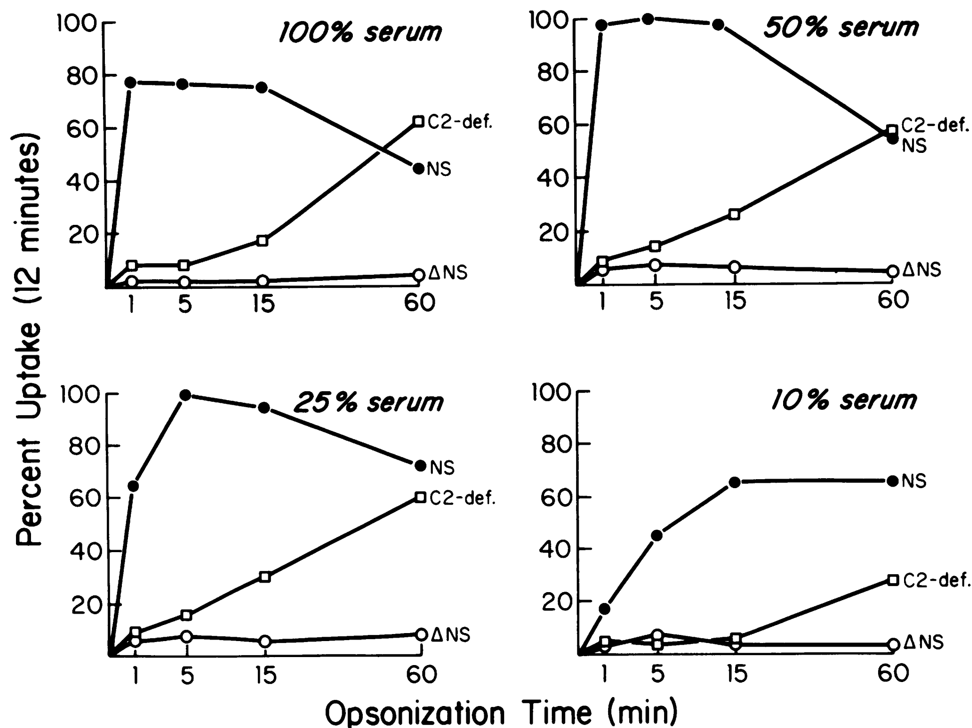

FIG. 3. *Phagocytosis of E. coli ON2 by PMN after incubation in human serum. Bacteria were opsonized for 1, 5, 15, and 60 min in 100, 50, 25, and 10% concentrations, respectively, of normal serum (●), heat-inactivated normal serum (○), and C2-deficient serum (□) before presentation to PMN. Phagocytosis was quantitated by measuring leukocyte-associated radioactivity at 12 min.*

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