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In Vivo Changes in Complement Induced with Peptidoglycan-Polysaccharide Polymers from Streptococcal Cell Walls

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In rats injected with an arthropathogenic dose of streptococcal cell wall fragments, serum hemolytic activity decreased over the first 24 h and was then elevated from days 2 through 6 after injection. Hemolytic activity was again elevated at days 26 and 40. Levels of activity of alternative complement pathway, C3, and factor $\overline{D}$ were also altered.

We have previously reported that the peptidoglycan-polysaccharide complex (PG-APS) isolated from cell walls of group A streptococci can activate the alternative complement pathway (ACP) in human serum in vitro (7). All of this activity is associated with the peptidoglycan moiety (7). It is now apparent from numerous reports that activation of ACP is a property of many bacterial species, and several components of bacterial cells have been identified with this activity (6, 19). We found that peptidoglycan is the most potent activator of the ACP (7).

Activated complement components function as mediators in inflammatory diseases (1, 5, 16) and are also involved in the regulation of immune responses (10, 14). Therefore, we investigated activation of the ACP induced in vivo by PG-APS to determine the role of complement components in the pathogenesis of experimental arthritis and immune dysfunction in the rat (4, 8).

Veronal-buffered saline (pH 7.5) with 0.1% gelatin (GVB), GVB plus 40 mM ethylenediaminetetraacetate (GVB-EDTA), and GVB plus 8 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid and 2 mM Mg^{2+} (GVB-MgEGTA) have been described (13).

Total serum hemolytic complement activity was measured as previously described (7), using sensitized sheep erythrocytes and a kinetic method devised by Boackle et al. (2).

Activation of the ACP in rat serum was measured by a modification of the method of Coonrod and Jenkins (3). Fresh rabbit erythrocytes were collected in Alsever solution from New Zealand white rabbits, and the buffy coat was removed. The erythrocytes were washed three times in GVB-MgEGTA and adjusted to a concentration of 5×10^8 per ml. A total of 20 μl of test serum was mixed with 65 μl of GVB-MgEGTA. Erythrocytes (20 μl) were then added, and the mixture was incubated for 20 min at 37°C. To provide an excess of terminal components and to ensure that cells which had interacted with the ACP were lysed, 40 μl of normal pooled rat serum diluted 1:1 with GVB-EDTA was next added to each tube. The reaction was allowed to continue at 37°C for another 20 min, after which the tubes were placed in an ice bath, and 1 ml of cold GVB-EDTA was added. The tubes were clarified by centrifugation, and the optical density was read at 412 nm. Controls included a cell control containing all reactants except serum, a control of 100% lysis (H_2O), and a control of lysis by normal pooled rat serum.

Factor $\overline{D}$ activity of rat serum was measured by the lysis of rabbit erythrocytes in the presence of factor $\overline{D}$-depleted human serum. Factor $\overline{D}$-depleted human serum was prepared by chromatography on BioRex 70 (20). A total of 10 μl of test serum was mixed with 20 μl of the factor $\overline{D}$-depleted serum plus 60 μl of GVB-MgEGTA. Erythrocytes (20 μl) were then added, and the mixture was incubated for 15 min at 37°C. The reaction was stopped by the addition of GVB-EDTA, and the percentage of lysis was determined by spectrophotometric absorbance at 412 nm. Controls included tubes containing all reactants except test serum, a control of 100% lysis (H_2O), and a control of lysis by normal pooled rat serum.

C3 levels in rat sera were estimated by radial immunodiffusion (12) with antibody specific for rat C3 (Cappel Laboratories, Cochranville, Pa.).

Purification of cell wall fragments (PG-APS) from group A streptococcal cells has been described (7, 8). Female Sprague-Dawley rats weighing an average of 150 g were obtained from Zivic-Miller, Allison Park, Pa. In each experiment, 10 rats were injected intraperitoneally with an arthropathogenic dose (60 μg of rhamnose per g of body weight) of fragments of PG-APS suspended in phosphate-buffered saline (pH 7.2) (4, 8). Ten control rats were injected intraperitoneally with phosphate-buffered saline. Rats were bled from the tail under light ether anesthesia before injection and at periods of 6 h to 52 days after injection. Blood was collected on ice, and serum was obtained 30 min after clotting at room temperature and was then stored at −70°C until complement analyses.

The changes in total serum hemolytic complement activity at intervals after intraperitoneal injection of PG-APS are shown in Fig. 1. The mean value of the hemolytic activity of the PG-APS-injected group was compared with the mean of the control group at each time interval. The mean for the control group is the 100% value at each interval and is shown by the horizontal line in Fig. 1. Samples of the standard pool of rat serum were also measured at each analysis. The range of hemolytic activity of the control rats was 35 to 195% of the standard serum pool. The range of the PG-APS-injected group was 4 to 374% of the standard pool.

The decrease in hemolytic levels over the first 24 h after the PG-APS injection was expected from the capacity of PG-APS to activate the ACP in vitro (7). The change from depressed to increased levels of hemolytic activity was apparent at day 2 after the injection, and significantly increased levels were maintained through day 6. Of even greater interest was the recurrence of significant increases at days 26 and 40 after the single intraperitoneal injection of PG-APS (Fig. 1).

Measurement of the components of the ACP (Fig. 2) gave results similar to the changes in total hemolytic activity. Further analysis of components of the ACP showed that the levels of C3 and factor $\overline{D}$ changed in an analogous pattern, although the increase of factor $\overline{D}$ was not significantly different from control values (Fig. 2).

The increase of ACP activity after initial depression has been reported in mice given multiple injections of cobra venom factor (9). Infection of both malnourished and normal rats with *Staphylococcus aureus* has been reported to induce a temporary elevation in the classical pathways and in the ACP 2 or 3 days after infection (15). To our knowledge, the pattern of recurrent increase of complement activity over a period of 40 days after a single injection of a nonviable agent has not been observed previously.

Human peripheral monocytes (21) and mouse peritoneal macrophages (11) can synthesize the complement components required for functioning of the classical pathway or the ACP. The PG-APS fragments can persist within macrophages, in vivo or in vitro, for prolonged periods and stimulate the macrophages to become activated as shown by cytotoxicity (17, 18) and enzyme secretions (16). Therefore, we propose that the increase in serum levels of complement activity after injection of rats with PG-APS reflects the stimulation of macrophages to synthesize complement proteins.

The elevated levels of serum complement activity (Fig. 1) correspond to episodes of inflammatory joint lesions in the model of experimental arthritis induced in rats with aqueous suspensions of PG-APS (4, 8). However, there is

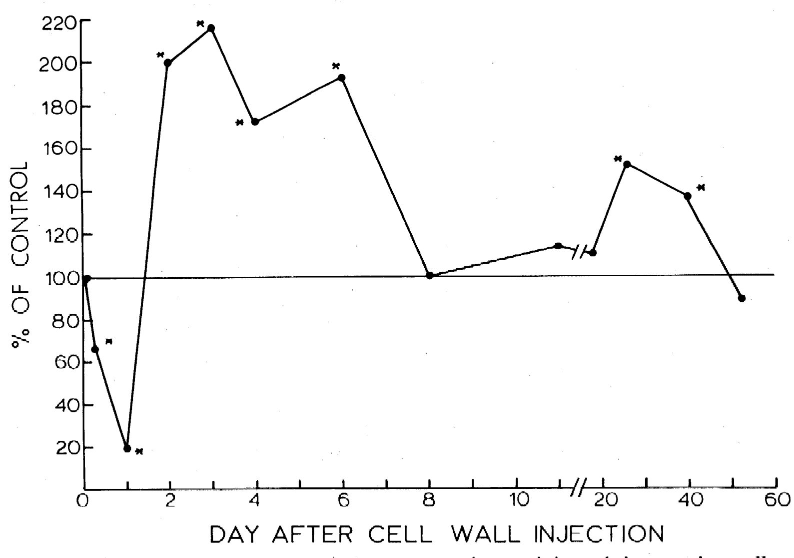

FIG. 1. Hemolytic complement activity in the serum of rats injected intraperitoneally with PG-APS, expressed as percentage of normal control serum. Each point is the mean of 10 rats compared at each time interval with the mean of 10 control rats by Student's t test. *, Significant difference of means at $P < 0.001$.

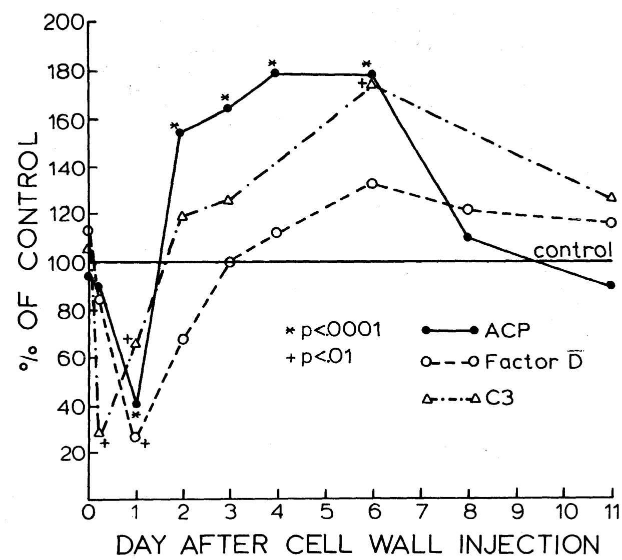

FIG. 2. Serum levels of ACP, factor D̄, and C3 in the serum of rats injected with PG-APS, expressed as percentage of control serum. Each point is the mean of 10 rats compared with the mean of 10 control rats by Student's *t* test.

no direct evidence, as yet, that these events are causally related, since there is no correlation between the severity of joint disease and the level of serum complement in individual rats.

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