INFECTION AND IMMUNITY, Jan. 1982, p. 377-380
0019-9567/82/010377-04$02.00/0

Vol. 35, No. 1

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

JOHN D. LAMBRIS,[1] JANICE B. ALLEN,[2] AND JOHN H. SCHWAB[2]*

Departments of Medicine[1] and Bacteriology and Immunology,[2] School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

Received 4 June 1981/Accepted 25 August 1981

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 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 D activity of rat serum was measured by the lysis of rabbit erythrocytes in the presence of factor D-depleted human serum. Factor 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 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

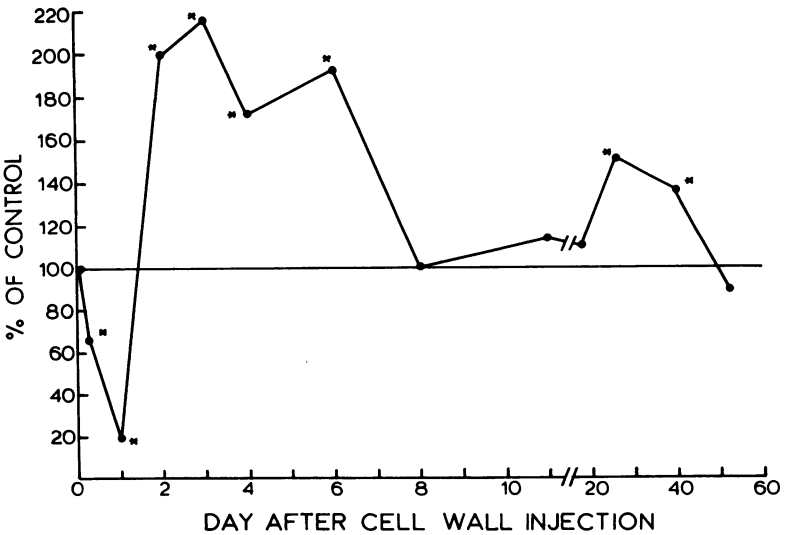

FIG. 1. Hemolytic complement activity in the serum of rats injected intraperitoneally with PG-APS, expressed as percentage of normal control serum. Each point is the mean of 10 rats compared at each time interval with the mean of 10 control rats by Student's t test. *, Significant difference of means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

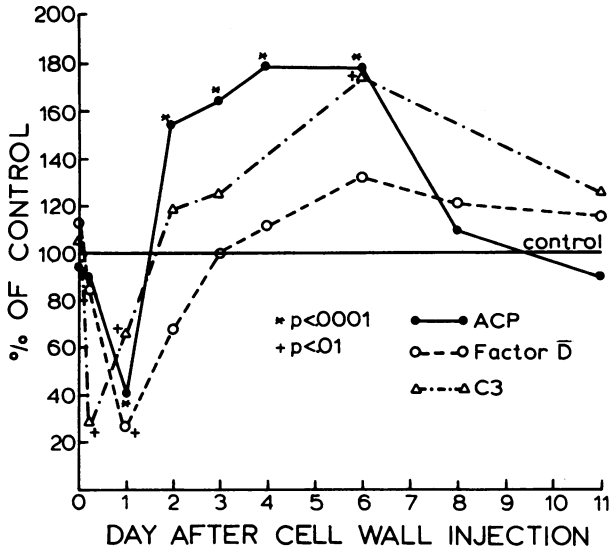

FIG. 2. Serum levels of ACP, factor $\bar{D}$, and C3 in the serum of rats injected with PG-APS, expressed as percentage of control serum. Each point is the mean of 10 rats compared with the mean of 10 control rats by Student's *t* test.

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.

This work was supported by Public Health Service grant AM25733 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

LITERATURE CITED

1. **Bellon, B.** 1979. Experimental immune glomerulonephritis induced in the rabbit with streptococcal vaccine. Clin. Exp. Immunol. 37:239–246.
2. **Boackle, R. J., T. M. Pruitt, and J. Mestecky.** 1974. The interactions of human complement with interfacially aggregated preparations of human secretory IgA. Immunochemistry 11:543–548.
3. **Coonrod, J. D., and S. D. Jenkins.** 1979. Functional assay of the alternative complement pathway of rat serum. J. Immunol. Methods 31:291–301.
4. **Dalldorf, F. G., W. J. Cromartie, S. K. Anderle, R. L. Clark, and J. H. Schwab.** 1980. Relation of experimental arthritis to the distribution of streptococcal cell wall fragments. Am. J. Pathol. 100:383–402.
5. **DeCeulaer, C., S. Papazoglous, and K. Whaley.** 1980. Increased biosynthesis of complement components by cultured monocytes, synovial fluid macrophages and synovial membrane cells from patients with rheumatoid arthritis. Immunology 41:37–43.
6. **Freudenberg, M. A., and C. Galanos.** 1978. Interaction of lipopolysaccharides and lipid A with complement in rats and its relation to endotoxicity. Infect. Immun. 19:875–882.
7. **Greenblatt, J., R. J. Boackle, and J. H. Schwab.** 1978. Activation of the alternate complement pathway by peptidoglycan from streptococcal cell wall. Infect. Immun. 19:296–303.
8. **Hunter, N., S. K. Anderle, R. R. Brown, F. G. Dalldorf, R. L. Clark, W. J. Cromartie, and J. H. Schwab.** 1980. Cell mediated immune response during experimental arthritis induced in rats with streptococcal cell walls. Clin. Exp. Immunol. 42:441–449.
9. **Joiner, K. A., A. Hawiger, and J. A. Gelfand.** 1980. The effect of cobra venom factor on alternative pathway hemolytic activity in mice. Immunol. Commun. 9:277–281.
10. **Jungi, T. W., and D. D. McGregor.** 1979. Role of complement in the expression of delayed-type hypersensitivity in rats: studies with cobra venom factor. Infect. Immun. 23:633–643.
11. **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.
12. **Mancini, G., A. O. Carbonara, and J. F. Heremans.** 1965. Determination of antigens by single radial immunodiffusion. Immunochemistry 2:235–254.
13. **Platts-Mills, T. A. E., and K. Ishizaka.** 1974. Activation of the alternative pathway of human complement by rabbit cells. J. Immunol. 113:348–358.
14. **Romball, C. G., R. J. Ulevitch, and W. Q. Weigle.** 1980. Role of C3 in the regulation of a splenic PFC response in rabbits. J. Immunol. 124:151–155.
15. **Sakamoto, M., S. Ishii, K. Nishioka, and K. Shimada.** 1981. Level of complement activity and components C1, C4, C2, and C3 in complement response to bacterial challenge in malnourished rats. Infect. Immun. 32:553–556.
16. **Schorlemmer, H. U., D. Bitter-Suermann, and A. C. Allison.** 1977. Complement activation by the alternative pathway and macrophage enzyme secretion in the pathogenesis of chronic inflammation. Immunology 32:929–940.
17. **Smialowicz, R., and J. H. Schwab.** 1977. Processing of streptococcal cell walls by rat macrophages and human monocytes in vitro. Infect. Immun. 17:591–598.
18. **Smialowicz, R., and J. H. Schwab.** 1977. Cytotoxicity of rat macrophages activated by persistent or biodegradable bacterial cell walls. Infect. Immun. 17:599–606.
19. **Verbrugh, H. A., W. C. van Dijk, R. Peters, M. van der Tol, and J. Verhoef.** 1979. The role of *Staphylococcus aureus* cell wall peptidoglycan, teichoic acid, and protein A in the process of complement activation and opsonization. Immunology 37:615–623.

20. **Volanakis, J. E., R. E. Schrohenloher, and R. W. Stroud.** 1977. Human factor $\overline{D}$ of the alternative complement pathway: purification and characterization. J. Immunol. **119**:337–342.

21. **Whaley, K.** 1980. Biosynthesis of the complement components and the regulatory proteins of the alternative complement pathway by human peripheral blood monocytes. J. Exp. Med. **151**:501–511.