Staphylococcal Clumping and Fibrinogen and Fibrin Degradation Products in Inflammatory Exudate[1] (35211)

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Staphylococci possessing clumping factor form clumps visible with the naked eye when mixed with a solution containing fibrinogen or its derivatives. Staphylococcal clumping factor is a polypeptide bound to the staphylococcal cell wall (1). As a paracoagulating agent distinct from staphylocoagulase, it interacts with fibrinogen, fibrin monomers and degradation products of fibrinogen and fibrin (1–4). By means of the staphylococcal clumping reaction it is possible to detect fibrinogen and its derivaties in biological materials. The reaction is very sensitive and permits one to detect as little as 0.03 μg of fibrinogen (4).

The clumping phenomenon may play a role in the development of experimental staphylococcal infection (5). Nonencapsulated strains of *Staphylococcus aureus* inoculated into the mouse peritoneal cavity are clumped and leukocytes adhere to the clumps of bacteria localizing the infection (5). However, the nature of the material responsible for the staphylococcal clumping reaction in inflammatory exudate remains undefined. It has been observed for some time that inflammatory exudate transferred from a body cavity into a glass tube forms a clot (6). This clotting may indicate that fibrinogen is present and that activation of the clotting mechanism has occurred. Morphologic evidence suggests that fibrin deposits at an inflammatory site are resolved (6–8). Therefore activation of fibrinolysis must occur in inflammatory exudates and degradation products of fibrin should also be generated. However, evidence for their presence is currently lacking.

The purpose of the present study was to determine what is the nature of the material causing staphylococcal clumping in inflammatory exudates and what its relationship is to polymorphonuclear leukocytes which constitute an important cellular component of inflammatory fluid. The data suggest that fibrinogen is not solely responsible for the staphylococcal clumping reaction in rabbit peritoneal exudate. Degradation products of fibrinogen and fibrin were demonstrated in the exudate and were also found in a subcellular fraction of exudative leukocytes. These degradation products contribute substantially to the clumping of staphylococci in inflammatory fluids.

Material and Methods. Rabbit peritoneal exudate. Exudate was induced in New Zealand white rabbits weighing 2.5–3.0 kg by the intraperitoneal injection of 0.15% glycogen in saline. Exudate was collected after 16 hr in citric acid–dextrose solution containing 360 mg of aminocaproic acid (EACA)/100 ml of exudate. In experiments involving proteolytic digestion of exudate by trypsin or plasmin EACA was omitted. The cellular portion of the exudate containing more than 95% PMN leukocytes was separated by centrifugation at 250*g* for 5 min, and the remaining supernatant was designated "peritoneal exudate." The cellular pellet was washed twice with modified Hank's solution containing glucose, albumin, heparin, and EACA. The pellet was then suspended in 7.5 ml of 0.34 *M* sucrose containing 24 mg of EACA and the cells were disrupted by sonication for 15 sec with a Branson Sonifier Model S110 equipped with a microtip 5.5 ×

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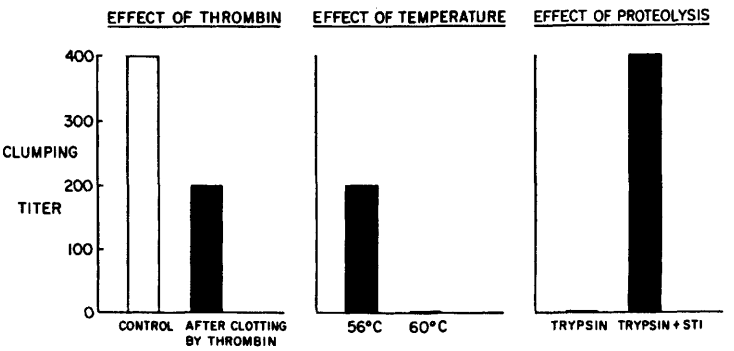

FIG. 1. Staphylococcal clumping in peritoneal exudate: Control represents native material with buffer. The effect of thrombin was examined after incubation of exudate with thrombin (100 U/1 ml of exudate) for 2 hr at 37° and separation of the clot. The effect of temperature was determined after heating of exudate as the given temperature for 10 min. The effect of proteolysis was examined after incubation of exudate with trypsin (0.2 mg/ml) at pH 7.4 for 1 hr at 37°. Soybean trypsin inhibitor was used in a concentration of 0.2 mg/ml.

0.125 in. and operated at 3 A. After sonication leukocytes were fractionated by differential centrifugation (9). Finally, the 8200*g* supernatant representing cytoplasmic constituents was designated as "the cytoplasmic fraction."

Staphylococcal clumping test. The presence of material reacting with staphylococcal clumping factor was determined quantitatively by the staphylococcal clumping test (slide method) according to the procedure described by Hawiger *et al.* (4).

Other methods. Double diffusion studies in agarose gel were performed according to the microtechnique described by Wadsworth (10). Fibrinogen was determined according to the method of Quick (11). Protein in trichloroacetic acid-insoluble fractions was determined by the method of Lowry *et al.* (12) with crystalline bovine albumin (Armour Pharmaceutical Co.) as a standard. Rabbit fibrinogen was obtained commercially from Nutritional Biochemicals Corp. Antirabbit fibrinogen serum was obtained from Cappel Laboratories, Dowingtown, Pa. The antiserum was adsorbed with normal rabbit serum according to the procedure described by Marder *et al.* (13). Thrombin (bovine origin) was obtained from Parke, Davis and Co. Plasmin was purchased from Novo Laboratories, Copenhagen, Denmark. Aminocaproic acid was obtained from Lederle Laboratories. Trypsin, 2× crystallized, was supplied by the Worthington Biochemical Corporation. Soybean trypsin inhibitor was supplied by Sigma Chemical Co.

Results. The experimental model for inflammatory exudate consisted of sterile PMN leukocyte-rich fluid collected from the rabbit peritoneal cavity. The protein content of the exudates averaged 120 mg/100 ml and the fibrinogen level of these exudates was approximately 30 mg/100 ml.

When staphylococci were added to peritoneal exudate, the clumping reaction occurred. Figure 1 shows the effect of thrombin, temperature, and proteolysis on the staphylococcal clumping titer in peritoneal exudate. The highest dilution of peritoneal exudate giving a positive clumping reaction averaged 400 (see Fig. 1, control). As noted, addition of thrombin lowered the clumping titer to one-half of its initial value. Removal of fibrinogen by thermal denaturation at 56° for 10 min caused a similar decrease in the clumping titer. This indicated that other materials besides fibrinogen were also responsible for staphylococcal clumping in peritoneal exudate. The remaining material was sensitive to heating at 60° and destroyed by the proteolytic action of trysin. Addition of soybean trypsin inhibitor (STI) to trypsin prevented the destruction of the material causing staphylococcal clumping.

To elucidate whether such material was derived from fibrinogen and/or fibrin, double diffusion tests with antirabbit fibrinogen

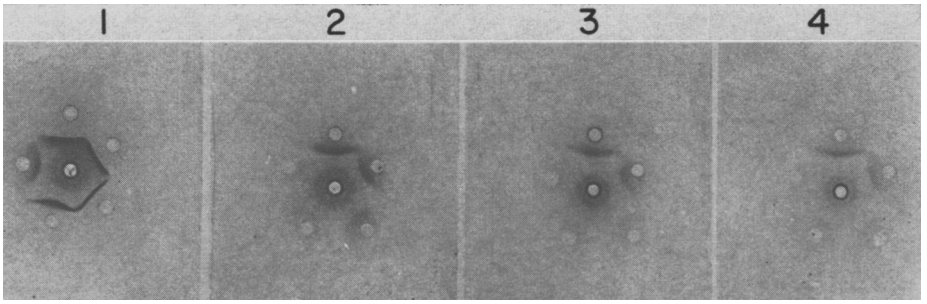

FIG. 2. Double diffusion in agarose gel with antirabbit fibrinogen: Antirabbit fibrinogen serum was applied in the center well and the test material around this in five different wells: undiluted, 1:2, 1:4, 1:8, and 1:16 (clockwise from top). Reactions were allowed to develop for 2 days at room temperature and then were made visible by staining with Buffalo black NBR. (1) rabbit fibrinogen; (2) peritoneal exudate; (3) peritoneal exudate treated with thrombin; (4) cytoplasmic fraction of PMN leukocytes.

serum were performed. As shown in Fig. 2, antifibrinogen serum reacted with 0.3% rabbit fibrinogen used as a control, when the fibrinogen solution was diluted up to 16 times. The reaction between peritoneal exudate and fibrinogen antiserum was less marked, occurring on the average at a 1:4 dilution of exudate. After addition of thrombin to peritoneal exudate and separation of clot, the remaining material was concentrated twofold by ultrafiltration and was tested with fibrinogen antiserum. Precipitation lines were obtained with this concentrated material. Thus, the non-clottable material in peritoneal exudate exhibited an antigenic relationship with fibrinogen.

Since polymorphonuclear leukocytes constitute the major cellular component of the peritoneal exudate employed in these studies, and such cells have been observed to phagocytize fibrin (7, 8), experiments were performed to ascertain whether fibrinogen derivatives like those formed in exudate were also present in the cytoplasm of exudative leukocytes. As shown in Fig. 3, a cytoplasmic fraction of PMN leukocytes contained material causing staphylococcal clumping but at a lower titer than exudate. Addition of thrombin to this material did not induce formation of a clot or change in the clumping titer. The substrate for staphylococcal clumping present in the cytoplasmic fraction of PMN leukocytes was partially sensitive to heating at 56° and inactivated at 60°. Proteolysis by trypsin completely destroyed it and plasmin partially digested it. Soybean trypsin inhibitor protected the material from proteolytic digestion by trypsin and plasmin. A cytoplasmic fraction of exudative leukocytes was tested for its reactivity with antirabbit fibrinogen serum in double diffusion tests. After fourfold concentration of the tested fraction a precipitation line was obtained indicating that the cytoplasmic fraction of exudative leukocytes contained fibrinogen derivatives similar to those present in peritoneal exudate (Fig. 2).

To demonstrate the specificity of the staphylococcal clumping reaction observed in the cytoplasmic fraction of PMN leukocytes, several clumping factor-positive strains and a clumping factor-negative strain were tested. Table I shows that the staphylococcal clumping reaction was positive only when strains possessing clumping factor were utilized. Staphylocoagulase did not contribute to the clumping phenomenon because strain Smith diffuse produces coagulase, does not

TABLE I. Specificity of Staphylococcal Clumping Reaction in a Cytoplasmic Fraction of PMN Leukocytes.[a]

Staphylococcal strain	Coagulase	Clumping factor	Clumping reaction
Newman	+	+	+
Newman D_2C	—	+	+
Smith diffuse	+	—	—
Zak	—	—	—

[a] +, positive; —, negative.

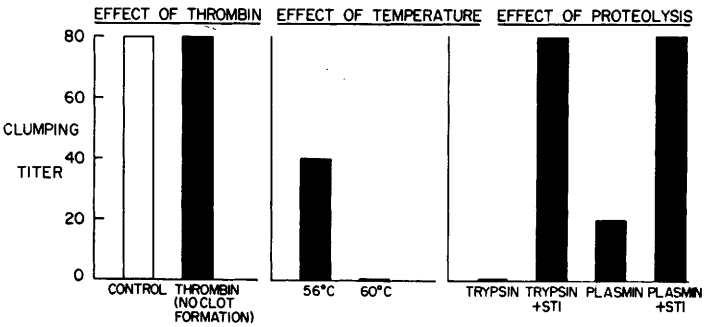

FIG. 3. Staphylococcal clumping in a "cytoplasmic" fraction (8200*g* supernatant) of PMN leukocytes. For details see Fig. 1. Concentration of plasmin was 0.5 mg/1 ml of fraction.

possess clumping factor, and was not clumped. It is thus apparent that the staphylococcal clumping phenomenon in a cytoplasmic fraction of PMN leukocytes is specific for staphylococcal clumping factor.

Discussion. Although the presence of fibrinogen and fibrin in inflammatory foci has been appreciated for a long time (6), the degradation products of fibrinogen and fibrin have not been previously shown as distinct components of inflammatory exudates. This was primarily due to the fact that fibrinogen and its derivatives possess similar antigenic determinants and a common ability to clump staphylococci. However, they differ in their clottability by thrombin and their sensitivity to temperature. These differences were utilized in the present study. The demonstration that rabbit peritoneal exudate contains in addition to fibrinogen, material not clottable by thrombin, but possessing antigenic determinants of fibrinogen, and causing staphylococcal clumping, indicates that this material represents degradation products of fibrinogen and/or fibrin.

It is known that staphylococcal clumping is caused by degradation products formed during the early stages of proteolytic digestion of fibrinogen and fibrin (4). Such products consist of Fragments X and Y (13). In contrast, degradation products obtained after prolonged digestion of fibrinogen by plasmin (Fragments D and E) do not clump staphylococci. However, such "late" products can form complexes with fibrin monomers which are not clottable by thrombin, but which can clump staphylococci (4, 14). Thus, it is conceivable that peritoneal exudate may contain several types of degradation products of fibrinogen and fibrin.

Fibrin degradation products are generated as a result of fibrinolysis. Active resolution of fibrin was observed in inflammmatory exudates by Opie in 1907 (6). He attributed this phenomenon to proteolytic enzymes called leukoproteases which he felt were released from polymorphonuclear leukocytes. Later it was found that PMN leukocytes were the source of plasminogen indicating that inflammatory exudates were well equipped for the local activation of fibrinolysis (15, 16). Thus, fibrin deposited in inflammatory foci can be degraded and may be engulfed by phagocytic cells. It has been shown by immunofluorescent techniques that PMN leukocytes engaged in the resolution of fibrin in inflammatory foci contain material reacting with antifibrinogen serum (7, 8). The present demonstration that fibrin degradation products are present in a cytoplasmic fraction of PMN leukocytes identifies this material as fragments of degraded fibrin, and provides further evidence that phagocytic cells actively participate in the degradation of fibrin.

Active phagocytic clearance of circulating fragments of fibrin has been also demonstrated in Kupffer cells of the liver (17, 18). Impairment of the phagocytic function of the reticuloendothelial system has been shown to lead to intravascular deposition of fibrin (19). A similar phenomenon may occur at local inflammatory sites when polymorphonuclear leukocytes are unable (possibly because of the deleterious effect of cytotoxic drugs, corticosteroids, or radiation therapy) to phagocytize degradation products of fibrin.

Moreover, destruction of PMN leukocytes may release strongly cationic lysosomal proteins which cause nonenzymatic precipitation of fibrinogen derivates (20) thus leading to secondary deposition of fibrin-like material in inflammatory foci.

Staphylococcal clumping reaction represents a valuable tool for the study of fibrinogen and fibrin degradation products in inflammatory exudates. The extent of staphylococcal clumping cannot be interpreted solely as a measure of fibrinogen content as preciously proposed by Duthie (1) and Kapral (5). Degradation products of fibrinogen and fibrin are present in peritoneal exudates, serve as substrates for the staphylococcal clumping reaction, and their level can be determined only after clotting of the fibrinogen in the exudate with a potent solution of thrombin. The presence of these degradation products of fibrinogen and fibrin causing staphylococcal clumping indicates that fibrinolytic mechanisms are functional in inflammatory foci.

Summary. Sterile peritoneal exudate induced in rabbits by infusion of glycogen was found to contain a material causing staphylococcal clumping. Removal of fibrinogen from the exudate partially diminished the clumping titer. The remaining active material was not clottable by thrombin, evoked a precipitation line with antifibrinogen serum in double gel diffusion tests, and was sensitive to heating at 60°. A similar material interacting with staphylococci possessing clumping factor was detected in a cytoplasmic fraction (8200*g* supernatant) of PMN leukocytes. These data indicate that rabbit peritoneal exudate contains not only fibrinogen but its derivatives. Such derivatives are also present in exudative leukocytes probably due to the phagocytosis and digestion of fibrin. The presence of degradation products of fibrinogen and fibrin in exudative fluid reflects local activation of fibrinolysis in inflammatory foci.

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1. Duthie, E. S., J. Gen. Microbiol. **13**, 383 (1955).

2. Allington, M. J., Brit. J. Haematol. **13**, 550 (1967).

3. Lipinski, B., Hawiger, J., and Jeljaszewicz, J., J. Exp. Med. **126**, 979 (1967).

4. Hawiger, J., Niewiarowski, S., Gurewich, V., and Thomas, D. P., J. Lab. Clin. Med. **75**, 93 (1970).

5. Kapral, F., J. Bacteriol. **92**, 1188 (1966).

6. Opie, E. L., J. Exp. Med. **9**, 391 (1907).

7. Riddle, J. M., and Barnhart, M. I., Amer. J. Pathol. **45**, 805 (1964).

8. Barnhart, M. I., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **24**, 846 (1965).

9. Cohn J. A., and Hirsch, J. G., J. Exp. Med. **112**, 983 (1960).

10. Wadswoth, C., Int. Arch. Allergy Appl. Immunol. **10**, 355 (1957).

11. Quick, A. J., Hemorrhagic Diseases and Thrombosis, 460 pp. Lea and Febiger, Philadelphia (1966).

12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

13. Marder, V. J., Shulman, N. R., and Carrol, W. R., J. Biol. Chem. **244**, 2111 (1969).

14. Marder, V. J., and Shulman, N. R., J. Biol. Chem. **244**, 2120 (1969).

15. Barnhart, M. I., and Riddle, J. M., Blood **21**, 306 (1963).

16. Prokopowicz, J., Biochim. Biophys. Acta **154**, 91 (1968).

17. Lee, L., and McCluskey, R. T., J. Exp. Med. **116**, 611 (1962).

18. Gans, H., and Lowman, J. T., Blood **29**, 526 (1967).

19. Lee, L., Prose, P. H., and Cohen, M. H., Thromb. Diath. Haemorrh., Suppl. **20**, 87 (1966).

20. Hawiger, J., Collins, R. D., and Horn, R. G., Proc. Soc. Exp. Biol. Med. **131**, 349 (1969).

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