Prevention of Dog Serum-Induced Aggregation of Pig Platelets[1] (36763)

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Transplantation antigens are present on the surface of platelets. This fact can be utilized for the detection of the corresponding antibodies (1) and for histocompatibility studies (2). It has been shown that platelet aggregometry is useful for studying the action of preformed antibody against these tissue antigens. Pig platelets were used as target cells and dog serum as source of the antibody since the dog has a powerful preformed humoral antibody against porcine tissue (3). It was assumed that the antigens on the surface of the pig platelets were the same as those of other porcine cells. Thus, the aggregation of pig platelets by dog serum was suggested to be a simple objective method to detect preformed antibodies to transplantation antigens and to serve as an *in vitro* model for xenograft rejection (4).

With the assumption that the foregoing conclusions are correct, platelet aggregometry with pig platelets as target cells and dog serum as aggregating agent can be considered to be a good tool for *in vitro* attempts to prevent the transplantation antigen–antibody reaction. In this communication such attempts are reported.

Two types of compounds were selected: (a) synthetic fibrinolytic agents, (b) semisynthetic pentosane polysulfo esters and related agents which exert anticoagulant activity (5). Complement inhibition is a common property of these synthetic fibrinolytic agents (6) and the pentosane polysulfo esters (5). The synthetic fibrinolytic compounds inactivate β_1C-globulin (C3) (6) whereas the sulfonated pentosanes apparently inhibit C1-esterase (7).

Material and Methods. Synthetic fibrinolytic agents: Flufenamic acid Na was donated by Dr. C. V. Winder, Parke Davis Research Laboratories, Ann Arbor, MI. Pamoic acid was purchased from K & K Laboratories, New York. Pentosane polysulfo esters: These were sodium salts of sulfonated xylans of two different molecular weights: SP-54, mol wt 2000, Bene-Chemie, Munich, Germany, xylanester (8), mol wt approx 7000 (property Dr. von Kaulla); and chitosan polysulfate, donated by Dr. R. Straessle, Hoffman-La Roche & Co., Basel, Switzerland. Lipo-Hepin Darwin, 200 mg (20,000 units USP)/ml was the heparin source. Pentosane polysulfo esters and related compounds were dissolved in buffered saline (pH 7.4) in such a way that desired final concentration in the serum–plasma mixture was obtained by addition of 20 μl solution. Bovine corium collagen was provided by Dr. W. R. Thomas, Armour Comp., Kankakee, IL; 1 mg collagen (85% pure collagen as determined by hydroxyproline content) was suspended in 1 ml saline and homogenized; 20 μl were added to 1 ml reaction mixture. Cobra venom factor: crude venom of the Egyptian cobra *Naja haje* was partially purified by chromatography on DEAE-cellulose and 1 ml dog serum incubated for 30 min with 20 μl of an active (20 μl reduced CH_{50} of 1 ml fresh normal human serum below 5) fraction.

All glassware was siliconized by exposing it to pervapored silicone solution (General Electric drifilm SC-87) followed by careful washing and drying.

Preparation of pig platelet-rich plasma (PPRP) and pig platelet suspension: Unanesthetized cross-bred (Yorkshire/ Hamp-

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shire/Duroc) pigs, 8–14 mo old weighing 8–12 kg were placed on their backs and tied by their legs on a V-shaped restraining board. A 18-gauge medium bevel hypodermic needle attached to a 5-ml plastic syringe was inserted at a 45° angle above the clavicle close to the sternum and the subclavian vein was punctured. The blood was withdrawn with the two-syringe technique, the second syringe, containing 2 ml of 3.8% sodium citrate USP, was filled to the 20-ml mark. Citrate and blood were carefully mixed and the citrated blood spun for 5 min at 4° at 1500*g*. The resulting plasma had a platelet count between 600,000 and 1,000,000 platelets/mm^3 and was immediately used as platelet-rich pig plasma (PPRP). The pig platelet suspension was prepared from this plasma by centrifuging it for an additional 15 min at 4° at 1000*g*. The plasma was discarded and the platelet button carefully resuspended in approx 5–6 ml saline (with pH adjusted to 7.4) by blowing it from a fine pipette against the sedimented platelets avoiding air bubbles which would change pH and aggregability. The suspended platelets were then recentrifuged and the process of resuspending and recentrifuging repeated three times. The final suspension was adjusted with saline to a platelet count of 800,000 and immediately used.

Preparation of dog serum. Male German shepherds weighing 16–20 kg were exsanguinated by drawing the blood from the jugular vein or from a femoral artery. The blood was allowed to clot for about 1 hr in nonsiliconized glass bottles and spun for 10 min at 1500*g* at room temperature. The serum was stored frozen in 5-ml batches in glass tubes at —80°. One milliliter of serum should not produce fibrin traces in 1 ml bovine fibrinogen solution 0.4% at 37° within 1 hr (check for thrombin traces). Samples with appreciable hemolysis were discarded. The samples were thawed immediately before use. The dog serum was kept in ice water and the pig plasma at room temperature during each of the series of runs which required about 1.5–2 hr.

Complement inactivation in dog serum. The dog serum was incubated at 37° with the compounds for time intervals and at concentrations indicated in the legends of the figures. The synthetic fibrinolytic compounds were subsequently removed by dialysis, because at the concentration used they might damage the pig platelets. One milliliter of serum was dialyzed by stirring at 4° against 500 ml saline for about 15 hr. Removal of the compounds by dialysis does not restore the complement activity of the serum. As indicated in the legends, Ca^{2+} was added to the dialysate or to serum before aggregation. For the studies with the pentosane polysulfo esters and related compounds, the thawed dog serum was preincubated for 5 min with these compounds. In other experiments, the pentosane polysulfo esters were preincubated for 5 min with platelet-rich pig plasma before the dog serum was added. All control sera were treated identically but in the absence of the above compounds.

Complement determination. Total hemolytic complement was measured in 50% hemolytic units (CH_{50}) by the method of Mayer (9). The complement activity of dog serum was reduced to <5 CH_{50} units/ml after preincubation with 12 m*M* flufenamic acid Na or 7 m*M* pamoic acid Na. Exposure of the dog serum to xylanester for 5 min in higher concentrations eliminated the complement activity. The critical concentration was 0.03% resulting in a reduction to 0–16 units, controls being $\pm$ 30 units. A part of the complement determinations was carried out by Dr. P. Kohler, Division of Clinical Immunology, University of Colorado Medical Center, Denver.

Platelet aggregometry. The platelet aggregation was recorded by a dual-sample platelet aggregometer (model DP 247-C, Sienco, Morrison, CO). This instrument records two aggregations simultaneously; the two resulting superimposed curves are obtained by feeding the output of the cuvette-photocell circuit into a stripchart recorder (chart speed 7 mm/min) on a time-sharing basis. The output was alternated every 10 sec. This feature together with a common light source and a common stirring motor for both cells

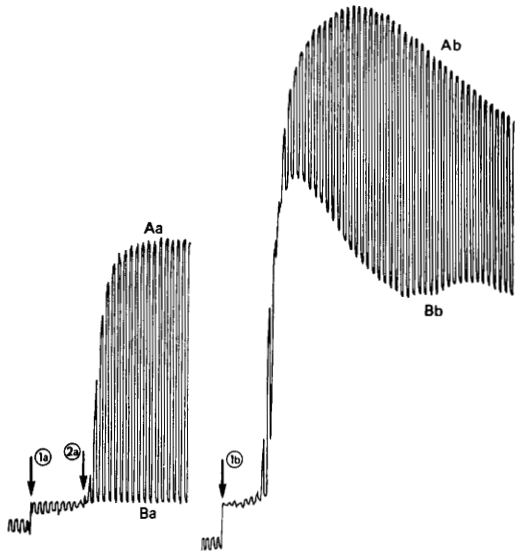

FIG. 1a. Complete prevention of dog serum (final concn 20%)-induced aggregation of pig platelets suspended in saline by flufenamic acid. (Aa) Dog serum dialyzed for 15 hr against saline at 4°. (Ba) Same as (Aa) except dog serum preincubated for 1 hr with 12 m*M* flufenamic acid. Inhibition 100%. (arrow 1a) Dog serum added to both samples; (arrow 2a) calcium chloride added to both samples, final concn 0.2 m*M*. Fig. 1b. Partial prevention of dog serum (final concn 20%)-induced aggregation of pig platelets in plasma by pamoic acid. (Ab) Dog serum incubated for 30 min at 37°, dialyzed 15 hr at 4° against saline containing 0.2 m*M* calcium chloride. (Bb) Same as (Ab) except dog serum is incubated for 30 min with 7 m*M* pamoic acid. Inhibition 41%. (arrow 1b) Dog serum added to both samples.

permits an accurate comparison of the two aggregation curves. These were obtained for the present study by twin samples (treated sample and control) from the same source. The aggregation inhibition is expressed in percent. The maximum aggregation height of the control is considered 100% and on this basis the percentage of the maximum aggregation height of the test sample is calculated. The difference is the aggregation inhibition (%).

Results. Dog serum in a final concentration of 20% aggregates both pig platelets in suspension and in citrated plasma very readily as shown in Figs. 1 and 2. With 10%, a clear-cut but less pronounced and delayed aggregation is obtained (not shown). The concentration of 20% was therefore chosen for this study. The reaction requires small amounts of calcium chloride. No aggregation was obtained when dialyzed dog serum without readded Ca^{2+} was used. No $CaCl_2$ was required with the nondialyzed sera in the studies with pentosane polysulfo esters and related compounds. The dog serum-induced aggregation of PPRP begins after a lag phase of 63 ± 12 sec (39 experiments) in the first run, it increases to 76 ± 13 sec (35) in the second run, to 89 ± 18 sec (30) in the third and to 232 ± 33 sec (22) in the fourth. The time elapsed between the beginning of the first and the fourth run was between 45 and 90 min. For reason of uniformity (possible difference of antibody titer), sera of only two dogs were used for the study. There was always an excellent aggregation of pig plate-

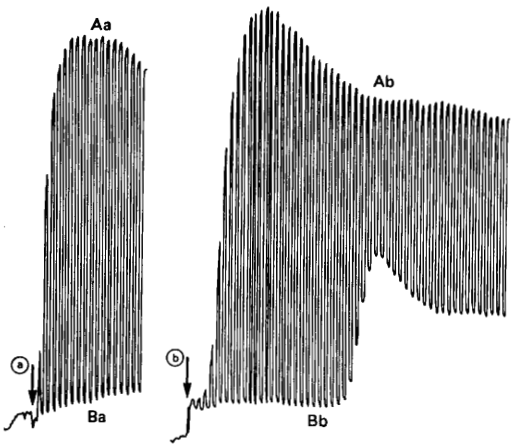

FIG. 2a. Prevention of dog serum (final concn 20%)-induced aggregation of pig platelets in plasma by SP-54. (Aa) Dog serum contains no SP-54. (Ba) Dog serum preincubated at 37° for 5 min with SP-54 to make a final concentration of 0.04%. Aggregation inhibition 96%. (arrow a) Dog serum added to both samples. Fig. 2b. Partial prevention of dog serum (final concn 20%)-induced aggregation of pig platelets in plasma by xylanester. (Ab) Dog serum contains no xylanester. (Bb) Dog serum preincubated at 37° for 5 min with xylanester to make a final concentration of 0.04%. Note aggregation delay in (Bb); aggregation inhibition 61%. (arrow b) Dog serum added to both samples.

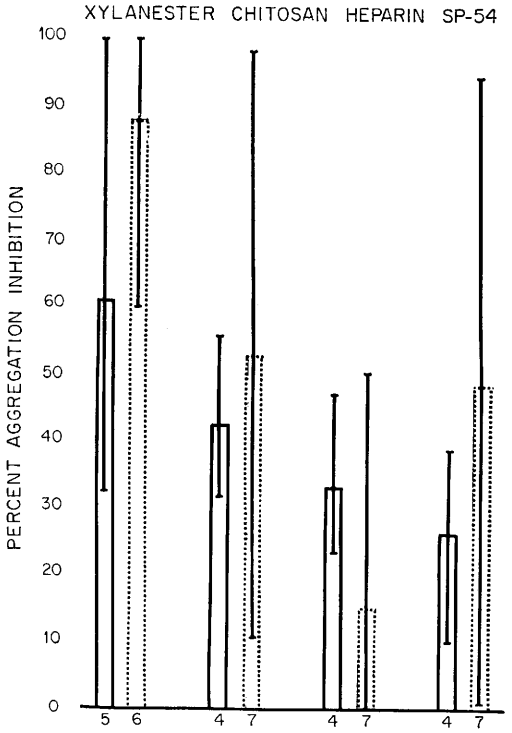

FIG. 3. Inhibition in percentage of dog serum- and collagen-induced aggregation of pig platelets in pig plasma by xylanester, chitosan, heparin and SP-54. (histograms) Average aggregation inhibition in percent (agregation of control: 100%). Number of experiments indicated below histograms. (lines in center of histograms) spread of values (percentage inhibition) [(—) histograms] dog serum-induced aggregation; [(- - -) histograms] collagen-induced aggregation.

lets in the 45 plasma samples tested although there was some difference in aggregation height as shown in Fig. 1. In order to ascertain that the observed aggregations with these two sera were not accidental, sera of seven more dogs were added to several PPRPs and good aggregations were obtained.

Elimination of complement activity of the dog serum by treatment with the synthetic fibrinolytic agent flufenamic acid blocked its ability to aggregate suspended pig platelets (Fig. 1a). Pretreatment with pamoic acid which equally abolishes the complement activity, reduced but did not inhibit completely the dog serum-induced aggregation of pig platelets in plasma (Fig. 1b). Pretreatment of the serum with the small pentosane polysulfo ester SP-54 appeared to abolish completely the dog serum capacity to aggregate pig platelets in PPRP (Fig. 2a). However, as seen in the following figures, there could be a misinterpretation of the results. Fig. 2b shows that the xylanester—the pentosane polysulfo ester with a larger molecular size—first appeared to abolish completely the dog serum ability to aggregate the pig platelets when indeed it initially prolonged the lag phase from 1 to 9 min and then produced an aggregation although inhibited by 61% (in the example shown). These and similar observations of a much delayed and only partially inhibited pig platelet aggregation by complement inactive dog serum are exceptions. In the majority of the pig plasma used, a more or less clear-cut aggregation inhibition is seen. Since only two dog sera were used for the study, the differences in response might have been due to the individual pig plasmas. In Fig. 3 the effect of xylanesters, SP-54, and of the two modified sulfonated hexoses (chitosan and heparin) were compared. The dog sera were preincubated for 5 min with 0.2% of the compounds. Their final concentrations in the serum–pig plasma mixture were 0.04%. Although there is a considerable spread of the values from pig plasma to pig plasma, Fig. 3 reveals clearly that the xylanester is the most effective agent and heparin the least effective one. With an increase of the xylanester concentration, the dog serum-induced pig platelet aggregation is more completely blocked. It suffices to add this compound 5 min before the dog serum is added to the pig plasma. Of six runs at 0.3% concentration, five resulted in a complete inhibition (100%) and one in 60% inhibition. At the 0.2% level, the values were 71, 88, 90, and 100% inhibition, and at 0.4%, there were 100, 100, and 95% inhibition. Heparin at the 0.4% level inhibited only at 31, 31, and 5%.

Figure 3 indicates furthermore that the four compounds (used at a higher final concentration of 0.15%) inhibit to some extent collagen-induced aggregation of platelets in pig plasma. Here again, the xylanester is the most effective one of the four compounds.

Preliminary experiments to probe into the role of complement showed the following: Preincubation of dog sera with Cobra venom factor resulted in 5 experiments with 3 different sera in aggregation inhibition of 82, 90, 91, 93, and 100%. Heating of the dog sera for 30 min at 56° gave in 5 runs aggregation inhibitions of 3, 4, 5, 20, and 75%.

Discussion. Various procedures have been tried in an attempt to modify the rapid rejection of xenotransplants, primarily pig kidneys transplanted into a dog. During the rejection, complement is activated and consumed; thus, attempts were made to suppress the complement activity in the host by cobra venom factor (10, 11), sodium hypochlorite (12), and intra-arterial citrate injection into the graft to remove Ca^{2+} required for complement activation (13). All these procedures prolonged the survival time of the graft to some extent. This effect could also be obtained by reduction of the platelet count of the host (14, 15). Furthermore, attempts were made to disrupt the chain of events which leads to rejection at an earlier stage by removing the dog anti-pig antibody by absorbing them with corresponding antigens from the donor tissue; a procedure which also prolonged the survival time of the graft (16). The present study confirms that dog serum readily aggregates pig platelets. This phenomenon is reminiscent of the fixation of antigen–antibody complexes on the thrombocytes, resulting in larger amounts of antibody in platelet aggregation. This reaction appears to be complement independent (17). It might be due to ADP release from the platelets which subsequently aggregate (18). Blocking of platelet aggregation induced by soluble antigens and antibodies in heparinized and citrated rabbit plasma by pretreatment with small amounts of C3-depleting cobra venom factor has also been described (19). Although in the present model, the antigens are thought to be fixed on the surface of the thrombocytes [as they are apparently on the surface of pig erythrocytes which are lysed by dog serum (20)], release of endogenous ADP secondary to the binding of the antibody has to be considered.

In this communication it is shown with an *in vitro* model, using pig platelet aggregation by dog serum, that the serum-induced platelet aggregation can be reduced or abolished altogether by certain compounds. The observed prolonged lag phase of dog serum-induced pig platelet aggregation, particularly with smaller concentrations of the inhibiting compounds tested, indicates that a time consuming reaction, required as a first step for the dog serum-induced pig platelet aggregation, is inhibited by the compounds. Whether or not complement inactivation plays a decisive role in the described blocking of the aggregation of pig platelets by dog serum is not clearly established by the studies reported here. It is true that xylanester concentrations (in the serum-PPRP mixture), for instance, which block complement activity completely or nearly completely, also block serum-induced platelet aggregation. However, there was also complete or near complete prevention of aggregation with xylanester concentrations which inhibited complement in the dog serum, but which were reduced to levels not sufficient for complete complement inhibition in the pig plasma–dog serum mixture after addition of the dog serum to the complement containing platelet-rich pig plasma. Furthermore, the fibrinolytic agents were removed by dialysis from the serum and therefore had no influence on the complement of the pig plasma. The complement activity in the mixture of PPRP with compound-treated serum was generally 15–20 CH_{50} and yet the aggregation was inhibited. Pilot experiments to obtain more information on the requirement of the complement gave no clear-cut results: Pretreatment of the dog sera with C3-depleting cobra venom factor reduced their aggregating ability for PPRP to nearly zero. Heating of the sera at 56° for 30 min, however, which resulted in a reduction of complement activity below 5 CH_{50}, diminished the ability to aggregate pig platelets of some sera but never abolished it. Further studies should solve the question of the role of complement and its components and also probe the possibility that the same mechanism by which the compounds prevent

collagen-induced platelet aggregation (21) is related to the prevention of dog serum-induced aggregation of pig platelets in plasma. It has been shown, for instance, that a great number of C′ inhibitors acting at various stages in the sequential interaction of C′ components also inhibit the platelet aggregation in human platelet-rich plasma by collagen (22).

From the practical point of view, the xylanesters and the less active SP-54 lend themselves to *in vivo* studies. Both have an anticoagulant effect which is clearly less than that of heparin. Heparin is efficiently anticomplementary at impractically high concentrations (23). The xylanesters are relatively nontoxic (8). They represent a particularly interesting group of semisynthetic sulfonated carbohydrates which are known to exert a more or less pronounced anticomplementary activity (5, 24). *In vivo* studies are likely to reveal whether or not the suppression of dog serum-induced aggregation of pig platelets has any bearing on the rejection of pig to dog xenografts.

Summary. Dog serum-induced aggregation of pig platelets in plasma has been considered to be an *in vitro* model for xenograft rejection. In this paper it is shown that this aggregation can be reduced or totally prevented by pretreatment of the dog serum with certain synthetic fibrinolytic agents or pentosane polysulfo esters or by adding the latter to pig plasma. The common denominator of these compounds is their complement suppressing activity.

1. Kissmeyer-Nielson, F., and Jensen, K. G., Progr. Clin. Pathol. **2**, 141 (1969).

2. Colombani, J., Transplant. Proc. **3**, 1078 (1971).

3. Rosenberg, J. C., Broersman, R. J., Bullemer, G., Mammen, E. F., Lenaghan, R., and Rosenberg, B. F., Transplantation **8**, 152 (1969).

4. Broersma, R., Mammen, E. F., Steiman, R., and Rosenberg, J. C., J. Surg. Res. **11**, 173 (1971).

5. von Kaulla, K. N., and Husemann, E., Experientia **2**, 222 (1946).

6. Aoki, N., and von Kaulla, K. N., Proc. Soc. Exp. Biol. Med. **130**, 101 (1969).

7. Walb, B., Loos, M., and Hadding, U., Z. Naturforsch. B **26**, 403 (1971).

8. Husemann, E., von Kaulla, K. N., and Kappesser, R., Z. Naturforsch. **1**, 584 (1946).

9. Mayer, M. M., *in* "Experimental Immunochemistry," 2nd ed. (E. Kabat, ed.), p. 133. Thomas, Springfield, IL (1961).

10. Moberg, A. W., Shons, A. R., Gewurz, H., Mozes, M., and Najarian, J. S., Transplant. Proc. **3**, 538 (1970).

11. Snyder, B. G., Ballesteros, E., Zarco, R. M., and Linn, B. S., Surg. Forum **17**, 478 (1966).

12. Bier, M., Zukoski, C. F., Boyer, J. T., Beavers, C. D., and Merriman, W. G., Trans. Amer. Soc. Artif. Intern. Organs **17**, 64 (1971).

13. Kux, M., Boehmig, H. J., Anemiya, H., Torisu, M., Yokoyama, T., Popovtzer, M., Launois, B., Wilson, C. B., Dixon, F., and Starzl, T. E., Surgery **70**, 103 (1971).

14. Slapak, M., Greenbaum, M., Bradawil, W., Saravis, C., Joison, J., and McDermott, W. C., Jr., Transplant. Proc. **3**, 558 (1971).

15. Derry, C. D., Kashii, A., Lafortune, L., P'eng, F.-K., Sayhoun, A., Misra, M., and Slapak, M., Trans. Amer. Soc. Artif. Intern. Organs **27**, 60 (1971).

16. Linn, B. S., Jensen, J. A., Portal, P., and Snyder, G. B., J. Surg. Res. **8**, 211 (1968).

17. Miescher, P., and Cooper, N., Vox Sang. **5**, 138 (1960).

18. Movat, H. Z., Mustard, J. F., Taichman, N. S., and Uriuhara, T., Proc. Soc. Exp. Biol. Med. **120**, 232 (1965).

19. Des Prez, R. M., and Marney, S. R., *in* "The Circulating Platelet" (S. A. Johnson, ed.), p. 415. Academic Press, New York (1971).

20. Rosenberg, J. C., Hawkins, E., Mammen, E. F., Palutke, M., Riddle, J., and Rosenberg, B. F., *in* "Coagulation Problems in Transplanted Organs" (K. N. von Kaulla, ed.), p. 107. Thomas, Springfield, IL (1972).

21. Thilo, D., and von Kaulla, K. N., J. Med. Chem. **13**, 503 (1970).

22. Jobin, F., and Tremblay, F., Thromb. Diath. Haemorrh. **22**, 469 (1969).

23. Di Perri, T., and Austeri, A., Soc. Ital. Biol. Sper. **46**, 817 (1970).

24. Ecker, E. E., and Pillemer, L., J. Immunol. **40**, 73 (1941).

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