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Rabbit Platelet Aggregation by Tuberculin[1] (39480)

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A wide variety of materials has been shown to induce platelet changes resulting in their aggregation and loss of internal constituents (1-3). Many of the substances investigated are capable of bringing about such changes by mechanisms involving direct interaction with a receptor site on the platelet surface. Other materials require the presence of complement, and in some cases, additional plasma cofactors for initiation of platelet aggregation and release reactions (4-7). The present communication reports platelet-aggregating activity by tuberculin, a substance which appears to irreversibly aggregate platelets via a complement-dependent reaction.

Materials and methods. Platelets. Blood was obtained by cardiac puncture from adult New Zealand white rabbits purchased locally. It was drawn into plastic syringes containing one-tenth volume of 3.8% trisodium citrate, or enough ethylene-diamine tetraacetic acid (EDTA) or ethylene-glycol tetraacetic acid (EGTA) to produce a final plasma concentration of 10 m*M* (calculated from hematocrit determinations). Platelet-rich plasma (PRP) was obtained after centrifugation of the blood at 150*g* for 20 min at room temperature. Platelets in PRP were counted in duplicate by the method of Stavem (8). For some experiments, albumin washed platelets were prepared according to the procedure of Walsh (9).

Tuberculin. Lot numbers 97477B and 974774L were obtained from Parke-Davis and Company, Detroit, Michigan. Company specifications of the lyophilized powder consisted of purified protein derivative 48.2% w/w, nucleic acid content 9.6% w/w and carbohydrate 42.2% w/w. Stock solutions of 1 mg/ml were prepared in saline.

Aggregometry. Platelet aggregation was examined turbidometrically on a Chronolog aggregometer (model No. 300, Chronolog Corp., Broomall, Pennsylvania) attached to a Heath Servo Chart Recorder (Model SR-201A). The baseline of the recordings was established with PRP adjusted to a concentration of 500,000 platelets/μl and the scale adjusted to give maximum deflection at the optical density of platelet-poor plasma (PPP). For studies of aggregation, PRP was placed in a siliconized cuvette and inserted into a temperature control holder with controlled magnetic stirring at 1200 rpm. A baseline was recorded for 5 min and the light transmission through the cell suspension recorded.

Complement inactivation and depletion. Heat inactivation of complement was accomplished by heating PPP to 50°C for 30 min. Heated plasma was centrifuged to remove precipitated fibrinogen prior to resuspension of fresh platelets. Hydrazine hydrate (Fisher Scientific Co., Medford, Massachusetts) adjusted to pH 7.5 was incubated with plasma at a final concentration of 5 m*M* for 60 min, the mixture dialyzed overnight at 4°C against 0.15 *M* NaCl with 5.5 m*M* sodium citrate (10) and added to fresh platelets. Plasma samples were incubated with four units of Cobra Venom Factor (CoF) at 37°C for 30 min. The purified material, the product of Cordis Laboratories (Miami, Florida), was suspended and diluted in distilled water immediately prior to use. Zymosan (Z) obtained from Sigma Chemical Company, St. Louis, Missouri, was preincubated with PPP at a concentration of 2 mg/ml (11) for 2 hr at 37°C.

Results. Recorder tracings of platelet aggregation by tuberculin. Typical aggregometry tracings of tuberculin-induced aggregation in stirred PRP are shown in Fig. 1. Aggregation responses were dose dependent for all concentrations. The first phase of the recorded reaction was a brief 1-2 min lag which diminished with increasing concentrations of tuberculin. During this period

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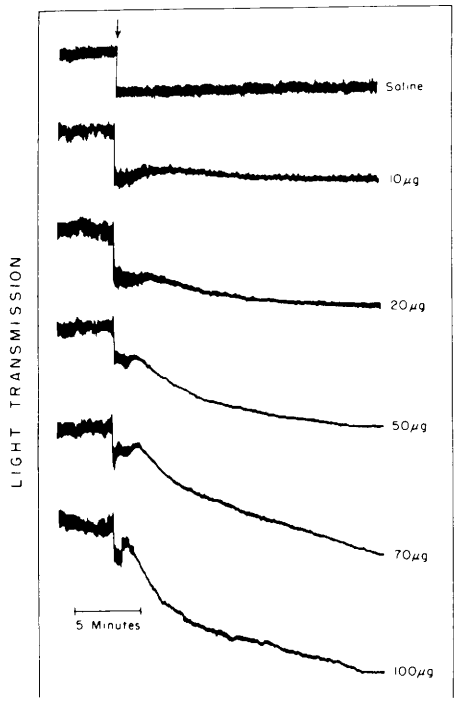

FIG. 1. Aggregometer tracings of tuberculin-induced aggregation in stirred citrated rabbit platelet-rich plasma. Tuberculin was added to a final concentration of 10, 20, 50, 70, and 100 μg per ml and a portion of the same plasma was inoculated with a similar volume of saline.

there was no change in either the slope of the curve or the vertical oscillations produced by the stirred discoid platelets. Microscopic examination of samples, however, revealed that small clumps consisting of 12–15 platelets had formed at this time. For tuberculin concentrations greater than 10 μg/ml, this phase was followed by an irreversible decrease in absorbance and reduction in amplitude of oscillations. Large aggregates with marked alterations in morphology were seen under phase contrast microscopy (Fig. 2). Many of the platelets contained in the clumps were transformed to irregularly swollen forms having greatly reduced refractility. Some of the platelets appeared to have fused and could not be identified as distinct cells. The morphological alterations were similar to those platelet changes collectively described as viscous metamorphosis (1). There was essentially no difference in sensitivity to tuberculin of platelets from different rabbits and aggregation was not observed in PRP incubated with saline. Platelet aggregation was not observed following addition of tuberculin to human citrated PRP prepared from venous blood.

Complement participation in the platelet response. The initial lag period between addition of tuberculin and appearance of platelet aggregation suggested an indirect action requiring plasma factors. Tuberculin failed to initiate aggregation when incubated with washed platelets suspended in Tyrode's solution. Addition of small amounts of PPP to the washed platelet suspension restored the aggregating activity.

The extent of complement participation was assessed by measuring aggregation of platelets in plasma which had been treated to inactivate or deplete complement (Table I). CoF, which inactivates C3–C9 by effecting reactions leading to the assembly of the alternate pathway C3 convertase (12), abolished the ability of the plasma to support an aggregation response. Chemical inactivation of C3 by pretreatment of plasma with hydrazine (13) similarly inhibited the reaction. Platelets suspended in plasma heated at 50°C for 30 min displayed minimal aggregation upon addition of tuberculin. A large degree of activity was restored by recombining equal volumes of heated and hydrazine-treated plasma. Removing proteins of the alternate complement pathway (ACP) by preincubation of plasma with Z (14, 15) abolished the ability of tuberculin to bring about platelet aggregation.

Differential chelation of divalent cations using EGTA was employed to help determine which complement pathway participated in the platelet–tuberculin interaction. EGTA–PRP provides appreciable concentrations of ionized magnesium but virtually no ionized calcium and is therefore capable of sustaining activation of the ACP while preventing action of the classical complement sequence (16). With EDTA as anticoagulant, the concentration of both calcium and magnesium ions is too low to permit activation of either complement pathway. Aggregometer tracings produced in EGTA–PRP and EDTA–PRP are shown in Fig. 3.

Anticoagulation with EGTA permitted a delayed aggregation response characterized by a lag ranging from 15 to 35 min. Aggregation was not observed in plasma anticoagulated with EDTA.

Discussion. Repeated experiments have demonstrated that tuberculin initiates agregation of rabbit platelets. The dependence of the reaction upon plasma factors is evidenced by the fact that aggregation does not occur when tuberculin is added to albumin washed platelets suspended in salt solution. A requirement for C3 is indicated by the absence of a response following addition of hydrazine. This treatment may abrogate the response by preventing complement dependent adherence reactions which are associated with aggregation of nonprimate platelets (17). In addition, either hydrazine or CoF may prevent aggregation by aborting the development of the lytic reaction mediated by terminal complement components.

Activation of C3 may occur via the classical complement pathway initiated by antigen–antibody complexes or by the ACP which can be activated *in vitro* by Z (15) and lipopolysaccharide (18). The present findings indicate that tuberculin can utilize the alternate pathway for the consumption of C3. Plasma previously heated to 50°C for 30 min fails to support a rapid aggregation response whereas a mixture of heated and hydrazine-treated plasma does. Heat and hydrazine-sensitive factors required for activation of the ACP by Z have been identified as glycine-rich β-glycoprotein (Factor B) and C3 (Factor A), respectively (19). The interaction of Z with human platelets has recently been shown to be a multisite reaction which also requires the presence of fibrinogen (14).

Platelets do not respond to tuberculin in EDTA–PRP whereas a delayed and diminished aggregation reaction was observed in plasma chelated with EGTA. It has previously been shown that in EGTA–PRP, immune compleses and Z induce a similar pattern of aggregation which was explained in terms of the action of the chelator (11). The concentration of magnesium in EGTA plasma is suboptimal for full activation of the ACP and the lack of ionized calcium would prevent aggregation reactions associated with the release of platelet adenosine-5′-diphosphate.

The inhibition of the aggregation reaction by adsorption with Z does not exclude the possibility that the response may also involve tuberculin activation of the classical complement pathway. Z has been reported to activate and consume C1–C4 (20) at a magnitude which may be sufficient to prevent tuberculin utilization of the classical pathway. Activation of C1 could occur by direct proteolytic cleavage or through interaction of tuberculin with complement fixing antibody. We have been unable to detect precipitating antibody by immunodiffusion, and tuberculin skin tests performed at the conclusion of the experiments were uniformly negative for immediate or delayed reactions. However, conventional serological procedures may not reveal the presence of low avidity antibody (21) and positive skin reactions do not correlate with the presence of antibodies to mycobacterial antigens (22). Although the rabbit is not a natural host for the tubercle bacillus, rabbit plasma may contain tuberculin-reactive immunoglobulin through immunization with cross-reactive antigens. In this regard, the sera of unimmunized and uninfected animals have been shown to induce immune adherence of *Mycobacterium tuberculosis*; the activity is interpreted as being due to low levels of natural antibody acting in conjunction with complement (17).

The present studies permit speculation that complement activation by antigens of the tubercle bacillus may be of some pathobiologic significance with respect to the development of tuberculosis lesions *in vivo*. There is a sizeable block of information that establishes a major role for fluid phase by-products of complement component interaction in the development of inflammatory lesions (23). The pathological changes which accompany tuberculosis in species bearing platelets sensitive to complement activation may be partially due to aggregation and release reactions. Rabbits, for example, consistently demonstrate thrombosis of small blood vessels during experimental

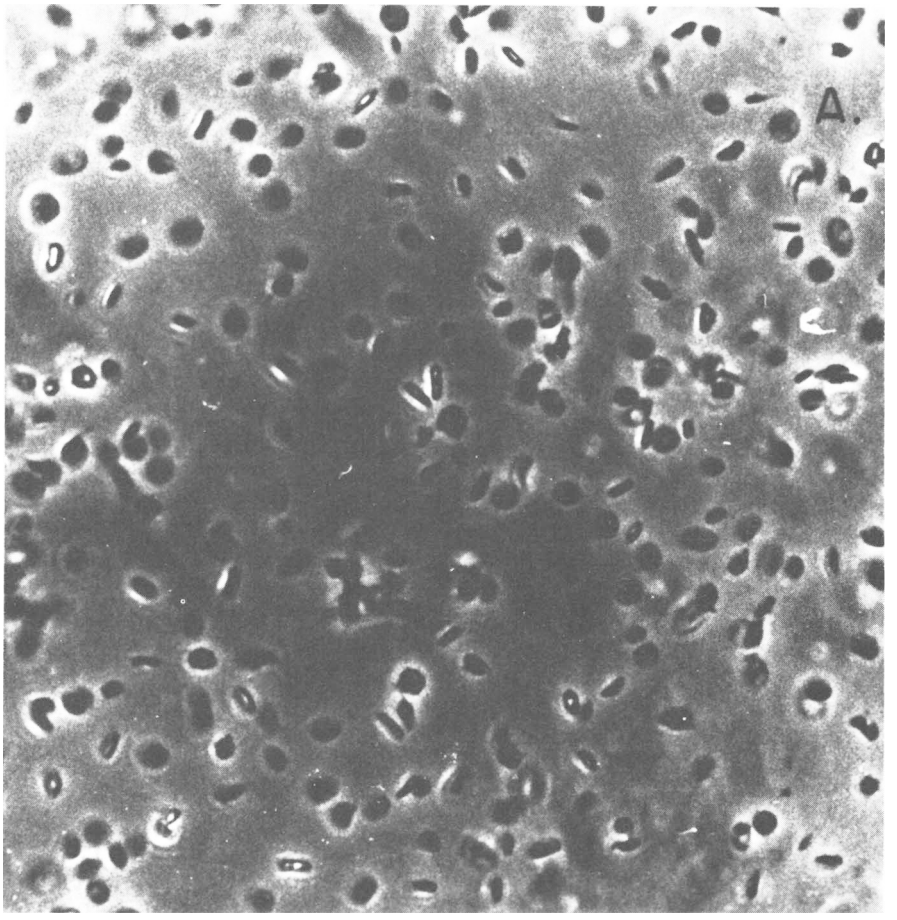

FIG. 2. Phase-contrast photomicrographs. A. Platelets in citrated plasma with saline, B. Platelets in citrated plasma incubated with 100 μg/ml tuberculin for 5 min at 37°C. Phase-contrast, ×1925.

TABLE I. THE EFFECTS OF VARIOUS PLASMA TREATMENTS ON THE ABILITY OF TUBERCULIN[a] TO INDUCE PLATELET AGGREGATION.

Plasma treatment	Platelet aggregation[b] (0 to ++++)
Untreated control	++++
Cobra venom factor (4 units)	0
Hydrazine (5 m*M*)	0
Heated (50 C/30 min)	+
Heated and Hydrazine (combined)[c]	+++
Zymosan[d]	0

[a] Treated plasma preparations plus platelets were incubated for 30 min with tuberculin at a final concentration of 100 μg/ml.

[b] Platelet aggregation was determined turbidometrically and by phase contrast microscopy.

[c] Plasma containing equal volumes of heated and hydrazine treated plasma.

[d] Zymosan was removed by sedimentation at 650*g* and platelets added.

tuberculosis (24). The induction by tuberculin of small platelet plugs could contribute to the development of thrombi and associated necrosis.

Summary. Tuberculin irreversibly aggregates rabbit platelets suspended in their native plasma. Phase contrast microscopy shows shape change and tightly packed aggregates. Inactivation of complement components by heat, hydrazine, cobra venom factor, and zymosan inhibit the reaction. Recombination of heat and hydrazine-treated plasma restored the capacity of tuberculin to initiate platelet aggregation. A delayed and diminished pattern of aggregation was observed in plasma chelated with EGTA while no response was observed in plasma anticoagulated with EDTA. The findings indicate that tuberculin can cause

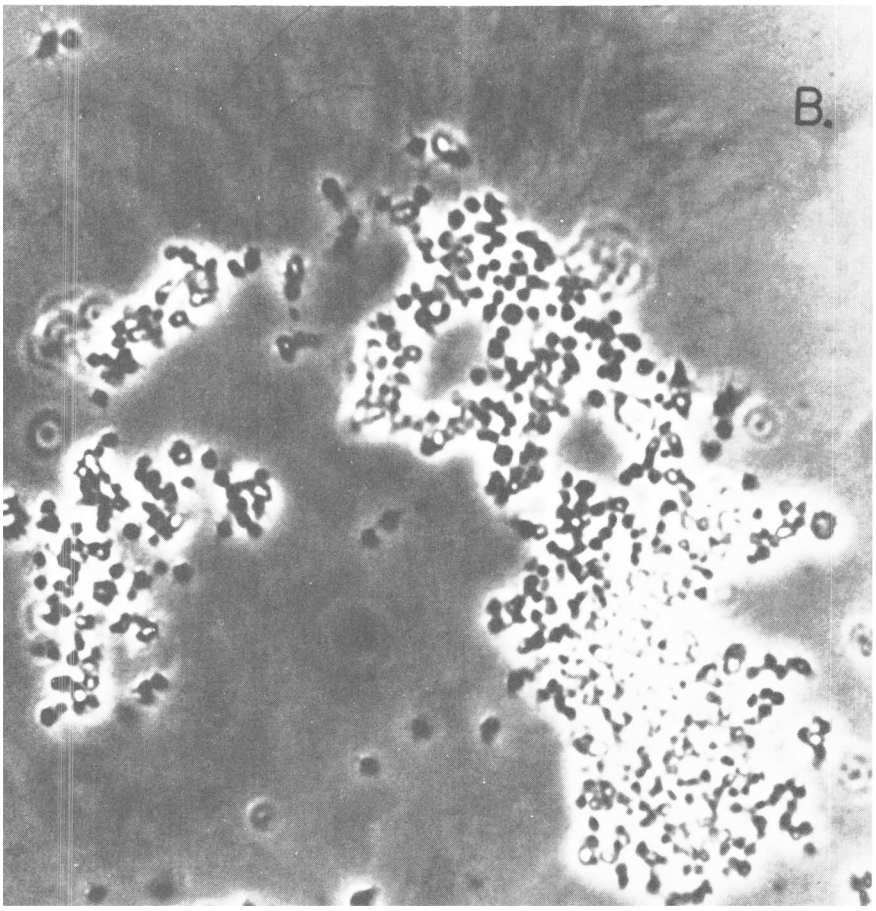

FIG. 2. *Continued.*

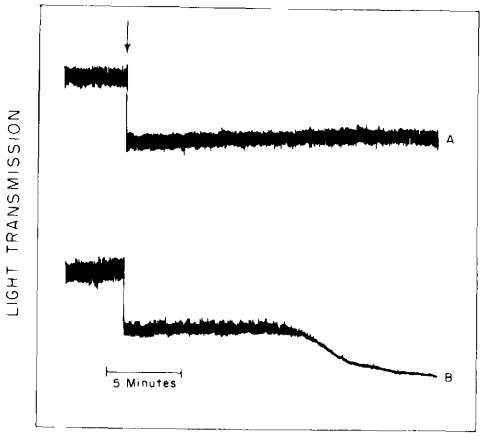

FIG. 3. Effect of chelation with EDTA and EGTA on tuberculin induction of platelet aggregation. Curve A: EDTA-PRP containing 100 μg/ml tuberculin. Curve B: EGTA-PRP containing 100 μg/ml tuberculin.

aggregation of rabbit platelets through a complement dependent mechanism, possibly utilizing the alternate pathway.

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