The Role of Protein Synthesis on the Generation of Tissue Factor Activity by Leukocytes (36346)

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Peritoneal leukocytes, obtained from rabbits which had been injected with endotoxin, had considerable procoagulant activity demonstrable both *in vivo* and *in vitro* (1, 2). To investigate whether human leukocytes could develop similar activity and to study the effect of inhibitors of protein synthesis *in vitro*, the development of coagulant activity was studied in incubation mixtures of leukocytes and endotoxin.

Evidence is presented below that human leukocytes incubated with endotoxin *in vitro* develop procoagulant (tissue factor) activity and that actinomycin D, puromycin, and cycloheximide inhibit the development of procoagulant activity.

Materials and Methods. Leukocytes were obtained from normal human blood anticoagulated with 0.1 vol of 4% trisodium citrate dihydrate. The red cells were sedimented with 0.3 vol of a 3% dextran solution [Dextran 250 (Pharmacia, NJ) dissolved in 0.15 *M* saline]. The leukocytes in the supernate were sedimented by centrifugation at 250*g* for 5 min. The sediment was resuspended in citrate saline[1] (5 parts 0.15 *M* saline, 1 part 4% trisodium citrate dihydrate) and the procedure was repeated 4 times. Remaining red cells were eliminated in some experiments after osmotic lysis (3). Their presence did not influence the results. The preparations contained 20,000 leukocytes and 2000–9000 platelets/mm^3. Platelet preparations containing up to 150,000 platelets/mm^3 and free of leukocytes did not generate any significant coagulant activity as measured by the one-stage test. A balanced salt solution containing dextrose but no protein (Hanks') was obtained from Difco (Detroit). Actinomycin D (Calbiochem, CA), cycloheximide and puromycin (Nutritional Biochemical, Cleveland,

[1] Ice cold citrate saline.

TABLE I. Tissue Factor Activity Generated by 2 × 10^6 Leukocytes.

The tissue factor activity (two-stage test) generated by leukocytes, in conditions indicated, was measured before and after 20 hr incubation at 37° (endot = endotoxin; ads ser = adsorbed serum).

	Tissue factor activity (μg) generated after incubation (hr)	
	0	20
WBC + ads ser + endot, 10 μg/ml	0	25
WBC + ads ser (no endot)	0	9*
WBC + Hanks' + endot, 10 μg/ml	0	14
WBC + Hanks' (no endot)	0	2*
WBC + ads ser + endot, 10 μg/ml + actinomycin D, 0.5 μg/ml	0	6
WBC + ads ser + endot, 10 μg/ml + cycloheximide, 0.25 μg/ml	0	3
Adsorbed serum + endot, 10 μg/ml	0	0

*Pyrogen-free glassware and reagents used.

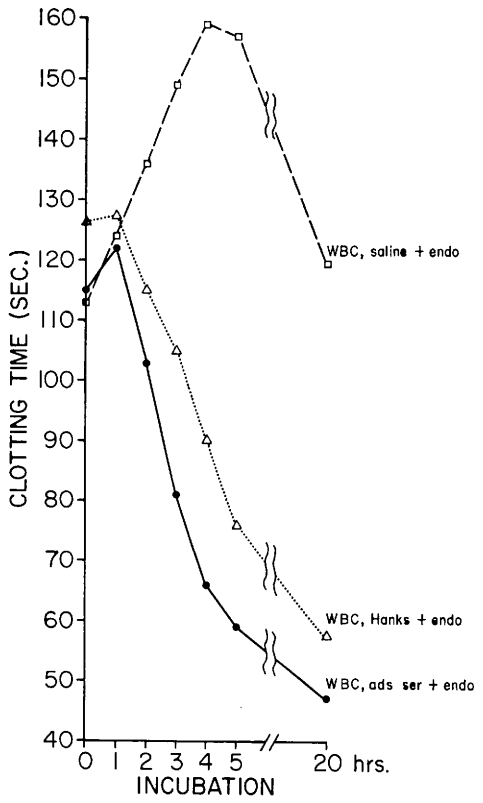

FIG. 1. Generation of procoagulant activity by leukocytes: Human peripheral leukocytes were incubated with endotoxin (10 μg/ml) and saline, or adsorbed serum, or Hanks' solution and the procoagulant activity generated was measured by the ability to clot normal plasma (one-stage test) (endo = *E. coli* endotoxin; ads ser = adsorbed serum).

OH) were dissolved in 0.15 *M* saline buffered at pH 7.3. Fresh suspensions of endotoxin *E. coli* 026B6 (Difco) were used at a final concentration of 10 μg/ml. Autologous human serum, obtained from blood allowed to clot at 37° for 2 hr (in glass) was adsorbed successively (twice, for 10 min) with 1% tricalcium phosphate (Baker) and the sediments were discarded. The adsorbed serum assayed by the method of Denson (4) was found to be free of factor X, and did not shorten the one-stage prothrombin time of a plasma deficient in factor VII.

The leukocytes were suspended in adsorbed serum, or 0.15 *M* saline, or Hanks' solution and incubated at 37° for 20 hr. Sterile glassware and reagents were used for the incubation mixture. The activity generated was tested at predetermined intervals.

The coagulant activity of the leukocytes was determined by a one-stage test as follows: 0.1 ml of citrated platelet-poor human plasma and 0.1 ml of leukocyte suspension were mixed; and the clotting time was measured after addition of 0.1 ml of 0.025 *M* $CaCl_2$.

The tissue factor activity was assayed by the two-stage method of Nemerson (5); and results obtained were compared with those obtained with a standard of commercial rabbit brain thromboplastin saline extract (Difco), which had a dry residue of 12.2 mg/ml. Results of tests on peripheral blood leukocytes from patients with chronic myelocytic leukemia (95% granulocytes), although not reported here, were similar. All results are the mean of at least three separate experiments.

Results. Leukocytes incubated in saline showed no procoagulant or tissue factor activity. Procoagulant and tissue factor activity,

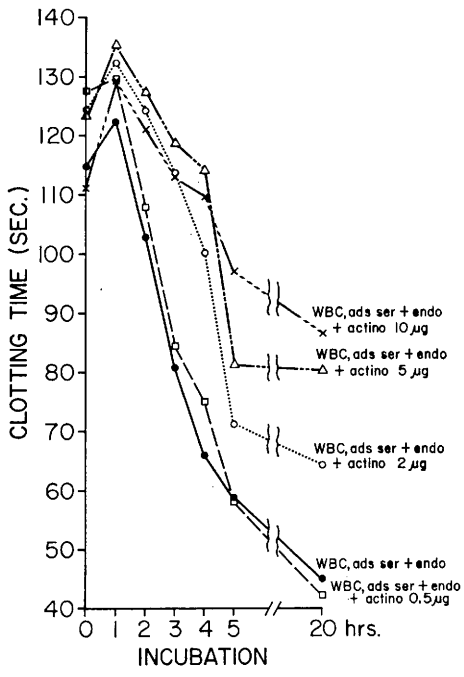

FIG. 2. The role of actinomycin D on the generation of procoagulant activity by leukocytes: actinomycin D, leukocytes, adsorbed serum, and endotoxin were incubated together; and the procoagulant activity generated was measured by the one-stage test (actino = actinomycin D).

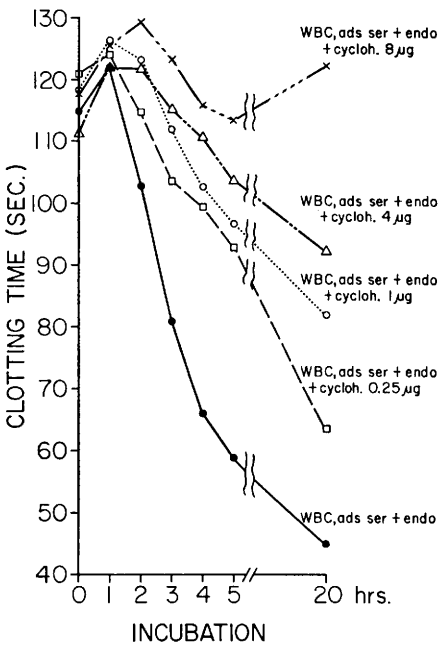

FIG. 3. The role of cycloheximide on the generation of procoagulant activity by leukocytes: cycloh = cycloheximide.

in increasing order, developed when leukocytes were incubated in Hanks' solution, adsorbed serum, adsorbed serum and endotoxin (Fig. 1, Table I). Maximal procoagulant activity was achieved after incubation of leukocytes for 18–20 hr with serum and endotoxin. These leukocyte suspensions shortened, similarly, the clotting time of normal or factor VIII- and factor IX-deficient plasma.

Twenty million cells incubated in adsorbed serum with endotoxin, yielded a tissue factor activity equivalent to 250 μg of tissue factor (Table I).

The protein synthesis inhibitors actinomycin D, cycloheximide, and puromycin added at the beginning of the incubation prevented development of coagulant activity of leukocytes incubated with serum and endotoxin (Figs. 2, 3, 4). However, when actinomycin D or cycloheximide were added after 2 or 3 hr of incubation no inhibition occurred (Table II).

Discussion. In the experiments presented, leukocytes generated procoagulant activity when incubated with adsorbed serum and endotoxin. Some of the findings in Fig. 1 are in agreement with the results presented by Lerner *et al.* (6). Lesser development of procoagulant activity occurred when leukocytes were incubated in Hanks' solution, thus suggesting that a serum factor is required for optimal activity. Endotoxin was necessary for optimal generation of the procoagulant activity.

Tissue factor activity was measured by a specific assay (5) and it is proposed that the procoagulant activity is due to tissue factor activity.

Of the protein synthesis inhibitors studied, actinomycin D inhibits DNA-directed RNA synthesis (7). Cycloheximide (8–10) and puromycin are protein synthesis inhibitors. As actinomycin D and cycloheximide did not suppress generation of the coagulant activity, when added after 2 hr of incubation, it is suggested that these antibiotics did not cause cell death and by suppressing protein synthesis prevented synthesis of the procoagulant activity.

If the procoagulant activity produced by 2

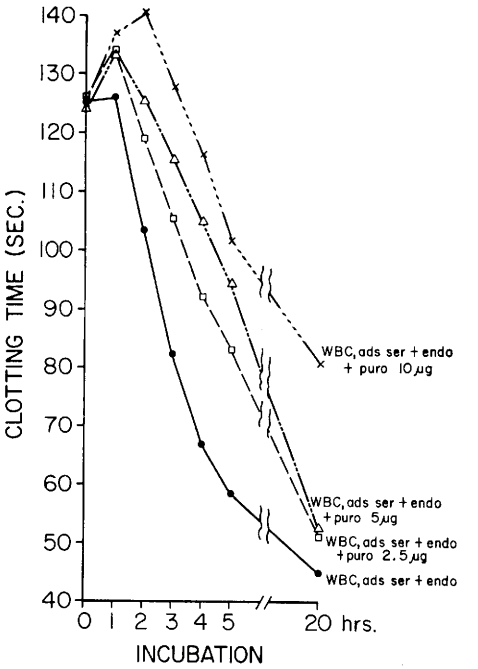

FIG. 4. The role of puromycin on the generation of procoagulant activity by leukocytes: puro = puromycin.

TABLE II. Effect of Actinomycin D and Cycloheximide on the Tissue Factor Activity.

Human leukocytes (20,000/mm³) were incubated with adsorbed serum and endotoxin. Actinomycin D or cycloheximide were added as indicated and the tissue factor activity assayed.

Drug added to the incubating leukocytes		Tissue factor activity (μg) generated 20 hr later
(μg/ml final)	After time (hr)	
Actinomycin D, 10	0	0
	2	21
Cycloheximide, 8	0	0
	2	22

$\times 10^7$ cells is related to the total body granulocyte pool, including cells from the promyelocyte to polymorphonuclear stage, *i.e.*, 1.2×10^{10} cells/kg of body weight (11), the potential activity produced (per kg of body wt) would be equivalent to about 150 mg of tissue factor or 12 ml of undiluted brain thromboplastin. This amount of thromboplastin could, conceivably, cause severe intravascular coagulation.

Summary. Peripheral leukocytes incubated with adsorbed serum and endotoxin generate procoagulant (tissue factor) activity. The generation of tissue factor activity is inhibited if actinomycin D, cycloheximide, or puromycin are added at the beginning of the incubation but no inhibition occurs if actinomycin D or cycloheximide are added after 2 hr incubation.

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