In vitro Inactivation of Rabbit Blood Thromboplastin by Macrophages.* (28279)

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Previous studies have provided evidence that blood thromboplastin is cleared from the circulation of an intact animal by reticuloendothelial phagocytosis(1). The data to be presented demonstrate that macrophages can remove thromboplastic activity from the environment in an *in vitro* system.

Materials and methods. All biological reagents were obtained from adult New Zealand male rabbits unless otherwise stated. Suspensions of macrophages were prepared by a modification of the method of Mackaness(2) as follows: Mineral oil (Nujol®) was sterilized by autoclaving for 1 hour, and was injected intraperitoneally in doses of 50 cc per animal. Four to 5 days after oil injection 100 cc of isotonic saline containing 10 mg of heparin were injected intraperitoneally, the animals were sacrificed with intravenous membutal, and the peritoneal cavity was laid open with a wide incision. The peritoneal fluid was collected by careful aspiration with plastic tubing attached to a siliconized syringe, transferred into siliconized test tubes and spun at room temperature for 5 minutes at 800 rpm in a clinical centrifuge. This procedure resulted in a sediment containing almost pure mononuclear cells; mineral oil and oil-laden macrophages rose to the top and were discarded with the supernatant fluid. The sedimented macrophages were then washed twice with Hanks' or Gay's solution and were finally suspended in the following medium to a final volume giving a cell count of about 20,000/cu mm.

	cc
Hanks' or Gay's Solution	75
$BaSO_4$—Adsorbed Rabbit Serum	5
.025 M $CaCl_2$	10
.1 M Glucose	10

The cells were used within 2 hours of collection, and their viability was verified by their ability to phagocytose Polystyrene Latex particles, 1.2 μ diameter, Dow Chemical Co.

Coagulation reagents, including sedimented blood thromboplastin and product I, were prepared as described by Spaet and Cintron (3); the source of crude cephalin was human brain(4). Radioactive crude cephalin was prepared from rat brain intrinsically labelled with P^{32}(1).

The basic experimental procedure was as follows: Each batch of peritoneal macrophages was divided into 3 aliquots. One of these was mixed with latex particles, and tumbled at 37°C to determine the viability of the cell suspension. The second was mixed with an equal volume of resuspended blood thromboplastin and similarly tumbled. The third was also mixed with blood thromboplastin, but was allowed to remain undisturbed for a similar period after initial mixing. An additional tumbled tube was prepared in which cell-free macrophage medium was substituted for macrophage suspension. Tumbling was accomplished for 30 minutes in stoppered test tubes attached to an upright circular rotator (Baltimore Biological Laboratories). Activity of the blood thromboplastin was estimated by its ability to accelerate the recalcified clotting time of normal human plasma(3). The thromboplastin in each specimen was measured immediately following ingredient mixing, after the 30 minute period, and again on the supernatant obtained following 5 minutes of centrifugation at 800 rpm. The activity in the cell-free specimen was taken as 100%, and dilution curves were made from it for use in determining activity of the others.

The same procedure was used to measure the ability of the cells to remove radioactive thromboplastin from the medium except that disappearance of radioactivity from the supernatant was determined instead of thromboplastic activity. Radioactive cephalin was rendered thromboplastic by exposure to product I(3). The effect of cells on the re-

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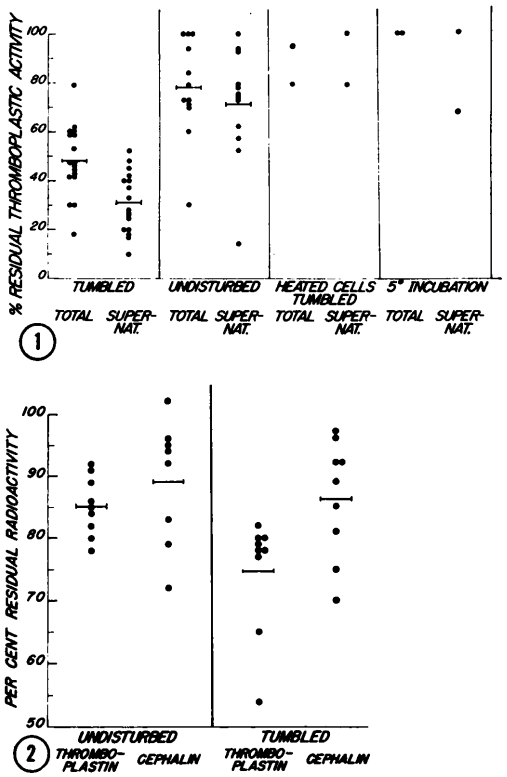

FIG. 1. Inactivation of blood thromboplastin by macrophages.

FIG. 2. Removal of radioactive thromboplastin and cephalin by macrophages. Radioactivity was measured on the supernatant after 30 min of incubation at 37°C and 5 min of centrifugation at 800 rpm. Radioactivity of cell-free specimen was taken as 100%.

sulting radioactive thromboplastin was compared to that on the same cephalin preparation without thromboplastic activity. The non-thromboplastic cephalin was processed identically but was exposed to coagulation-inert reagents instead of to product I(1). Radioactivity was measured by an end window Geiger-Muller tube.

Results. Immediately after mixing, all tubes gave clotting times of about 15 seconds with substrate plasma. The data presented in Fig. 1 show that thromboplastic activity decreased when intimate and continuous mixing of cells and reagent was accomplished at 37°C ($P = <.01$ as compared to the undisturbed control). In the cell-free system, slight loss of thromboplastic activity occurred, presumably due to inactivation by serum proteins; loss of activity in the cell-containing but undisturbed system was little different from that in the cell-free control. Heating the cells at 56°C for 5 minutes destroyed their ability to inactivate blood thromboplastin, and the inactivation system was similarly inert when the procedure was performed at 5°C. In systems which showed thromboplastin inactivation, cells exposed to latex instead of clotting reagent were almost all loaded with latex particles; systems which failed to inactivate thromboplastin showed little or no evidence of latex phagocytosis.

The data with radioactive cephalin were less impressive, although the direction of change was the same. As shown in Fig. 2, less radioactivity was found in the supernatant when cephalin with thromboplastic activity was used in the rotated system than with non-activated cephalin ($P = .02$), or with either reagent allowed to remain undisturbed ($P = .01$). At the centrifugal forces used, there was no detectable sedimentation of either cephalin preparation in the absence of cells.

An attempt was made to determine whether there was increased oxygen consumption by macrophages in the presence of blood thromboplastin. Incubation studies in the Warburg apparatus failed to reveal significant differences in oxygen consumption by cell suspensions after mixing with blood thromboplastin. However, even in control cell suspensions without blood thromboplastin, oxygen consumption was extremely rapid, and was not significantly increased by the addition of latex particles which were visibly phagocytosed. It was concluded that the cells prepared by the present technique were already at maximal metabolic activity. Pulmonary macrophages, prepared by the method of Myrvik, *et al.*(5) phagocytosed latex and showed significantly increased oxygen consumption in this process. However, they were unable to inactivate blood thromboplastin as tested by the methods described above, and did not show increased oxygen consumption in the presence of the coagulant.

The effect of peritoneal macrophages on product I was tested by mixing and determining residual clotting activity as above. No loss of activity was observed.

Discussion. The above data are in conformity with the view(1) that blood thromboplastin prepared *in vitro* is susceptible to reticuloendothelial phagocytosis. The cell-free control demonstrated that the thromboplastin was relatively stable in the suspending medium; the failure of undisturbed cells to inactivate thromboplastin indicated that an anticoagulant agent was not elaborated in the course of incubation. Measures that inhibited phagocytosis correspondingly reduced thromboplastin inactivation in the presence of the macrophages.

Failure of the cells to inactivate product I agrees with previous data(6) suggesting that this reagent is cleared *in vivo* with the participation of hepatic parenchymal cells rather than macrophages.

It is not evident why removal of radioactive thromboplastin was less effective than destruction of thromboplastic activity. Possibilities include: association of clotting activity with smaller particles, which might be more efficiently cleared than larger particles, resulting in a relatively low uptake of total lipid and radioactivity; digestion of phagocytosed material with release of radioactivity; or selective uptake from the crude cephalin of lipids known to have coagulant activity. The last explanation would fit with the relatively low content of phosphatidyl serine and ethanolamine (the coagulant lipids) in the P^{32} labelled crude cephalin(7).

All available studies are consistent with the view that laboratory blood thromboplastin is considered a foreign particle by the reticuloendothelial system. It remains to be determined whether clotting activity which forms under physiological conditions is similarly handled.

Summary. Under appropriate conditions rabbit peritoneal macrophages inactivated blood thromboplastin *in vitro*. In a similar system pulmonary macrophages were inert. The peritoneal macrophages appeared to remove radioactive thromboplastin from the medium, but the data were not as clear-cut. It was not possible to demonstrate increased oxygen uptake by peritoneal macrophages following exposure to thromboplastin since these cells appeared to have maximal oxygen consumption prior to mixing with the clotting reagent. It is concluded that reticuloendothelial cells treat laboratory blood thromboplastin as a foreign body.

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Effects of Freezing and Storage on Survival of Thyroid Autografts.* (28280)

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Freezing of the thyroid gland under various conditions has been reported(1,2) to be followed by survival of heterotopic autotransplants for periods up to 4 weeks. Stewart and co-workers(2) indicated that addition of

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