METHODS

Equipment and Technique for *In Vitro* Modeling of Thrombogenesis

I. P. Stepanova and S. P. Domogatskii

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The device is a centrally oriented rotary magnetic disk producing a certain clearance between the rotary part and the collagen-coated bottom of a standard multiwell plate. Regulation of the speed of rotation makes it possible to reproduce the high shear rates characteristic for large elastic arteries.

Key Words: *collagen; thrombogenesis; model of thrombogenesis*

Collagen is a basic component of the arterial wall which becomes exposed to the lumen after vessel injury. This is an adhesive substrate to which circulating platelets attach. One of the objectives of the present investigation was to develop a model of a damaged vessel wall with exposed collagen components. To this end we used surfaces precoated with thrombogenic type I-III collagen.

The well-known Baumgartner model of coagulation [2] consists of a perfusion chamber with denuded vessel segments fixed in the middle. The chamber is perfused with either whole blood or platelet-rich plasma (PRP), which simulates a certain rate of blood flow. The main disadvantages of this model are the tedious procedure of vessel preparation and difficulties involved in microscopic examination.

The method of probes rotating in a platelet suspension containing inverted denuded vessel segments, which was proposed by Cazenave *et al.* [3], also suffers from the above limitations. Moreover, global platelet activation in the suspension occurring due to an extensively damaged lumen surface, prevents the study of different stages of platelet-substrate interaction.

The Leitin model [1] is relatively simple: it consists of multiwell polystyrene culture plates with the bottom precoated with either fibrillar or monomeric collagen. Washed Cr-labeled platelets are added to the wells, and the plates are rotated in the horizontal plane at 36 rpm or rocked back and forth (20 cycles/min). Adhesion of the Cr-labeled platelets to the collagen-coated surface is studied as a function of time and dose. However, with this model it is impossible to achieve a high rate of rotation of the platelet suspension relative to the collagen substrate, i.e., to simulate coagulation conditions at high blood flow rates. The aim of the present study, therefore, was to develop a simple model of thrombogenesis, reproducing high rates of blood flow in vessels when collagen exposed to the lumen due to endothelium injury induces contact activation and aggregation of platelets and the formation of a parietal clot.

MATERIALS AND METHODS

The proposed model consists of the following components:

1) chambers, wells of culture plates (multiwell, Linbro) with an inner diameter of 16.4 mm. The

Group of Engineering Immunology, Cardiological Research Center, Russian Academy of Medical Sciences, Moscow. (Presented by V. N. Smirnov, Member of the Russian Academy of Medical Sciences)

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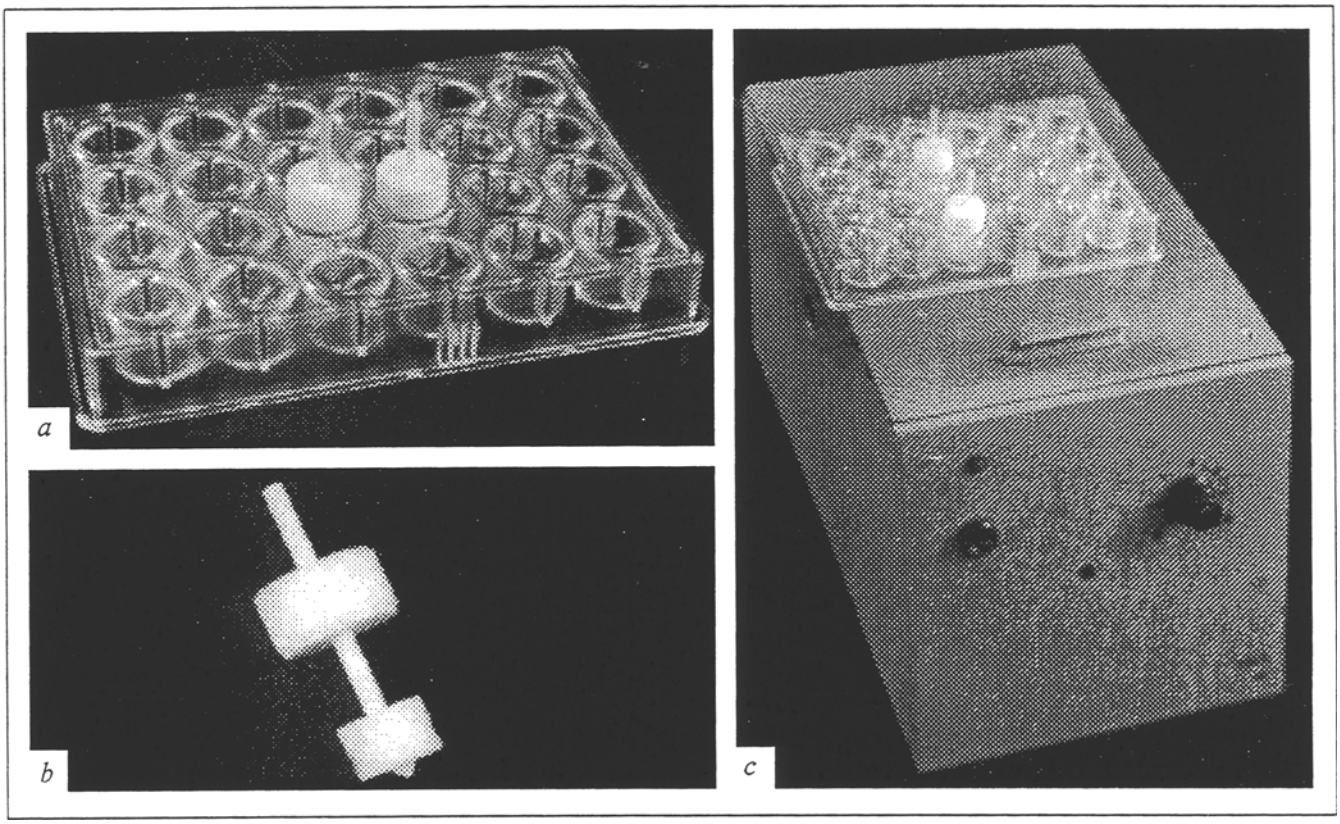


Fig. 1. Simultaneous use of several wells of culture plates as microchambers (*a*); magnetic stirrer agitating liquid in the wells (*b*); general view of the device for modeling thrombogenesis consisting of a magnetic field generator; multiwell culture plates, and magnetic stirrers (*c*).

plates are made of activated polystyrene, whose surface actively absorbs various proteins, including collagen. The use of culture plates as a surface for the immobilization of collagen has a number of methodological advantages: a) the multiwell structure of the plates makes it possible to manipulate 24 samples at once; b) the experiments require minimal quantities of collagen, platelets, and agents modulating their interaction due to the small size of the wells (Fig. 1, *a*).

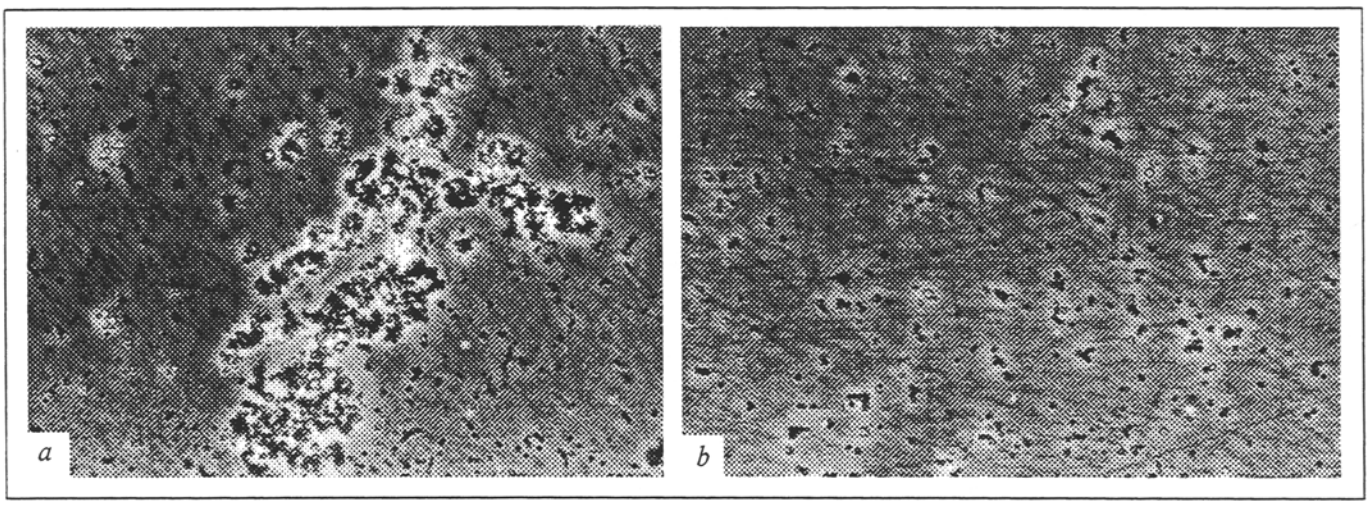
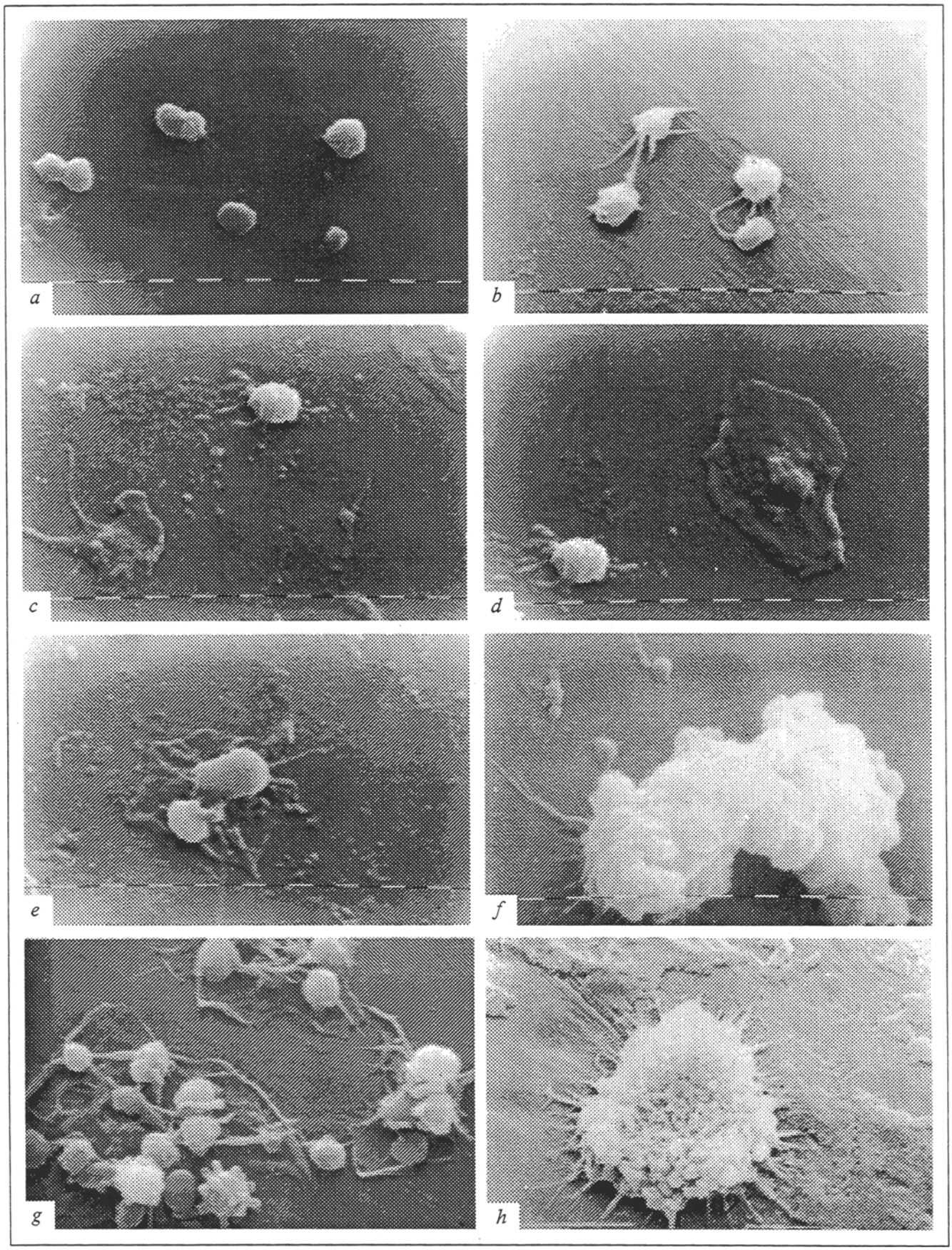


Fig. 2. Aggregation of platelets from PRP on collagen substrate (*a*); PRP after interaction with plastic well bottom (*b*). Phase–contrast microscopy, ×600.

Fig. 3. Interaction between platelets and plastic surface (*a*); formation of platelet pseudopodia (*b*); platelet adhesion (*c*); plating of platelet on collagen surface (*d*); adhesion of platelets from suspension to plated platelet surface (*e*); formation of platelet clot (*f*); organization of the clot with fibrin threads (*g*); clot retraction (*h*). Electron microscopy: *a*, *h*) ×2500, *b*–*g*) ×5000.


a
b
c
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h

TABLE 1. Interaction of Platelets with Collagen-Coated Bottom of Multiwell Plate.

Stage of platelet activation	Plastic	Collagen	Elapsed time, min
Formation of pseudopodia	–	+	3–5
Plating	–	+	5–7
Adhesion	–	+	10
Aggregation	–	+	12

2) 16-mm-long stirrers made of a hard magnetic material (Be-Fe-B) sealed in Teflon capsules. The stirrers rotate in a magnetic field and agitate biological fluids in the wells (Fig. 1, *b*).

Each stirrer rests on the well bottom with a thin fixing rod preserving it from damage during rotation. Further support is provided by a Teflon cover with holes for the stirrers.

3) a magnetic field generator in an insulating housing. The generator maintains simultaneous rotation of several stirrers in the wells, setting various rotation rates (0.5-10 rev/sec, Fig. 1, *c*).

Whole blood was drawn from the ulnar vein of volunteers and centrifuged at 1000 g for 12 min not later than 15 min after being drawn; 130 mM sodium citrate was used as an anticoagulant. The cell count was determined with an automatic platelet counter. It ranged from 4.5 to 5×10^8 cell/ml. Rat collagen, type I-III, in a concentration of 10 μg/ml, was absorbed in the wells. Collagen was extracted from rat tail tendon with 2% pepsin in 500 mM acetic acid, pH 2.5, purified, redissolved in acetic acid, and sedimented in acidic and neutral 1 M NaCl solutions.

One milliliter of PRP was placed in collagen-coated and non-coated wells. The stirrers provided an agitation rate of 1 rev/sec during 0-60 min, the shear rate at the bottom of the well being 500 sec^{-1}. Platelet aggregation on the collagen substrate as well as platelet-plastic interaction was assessed using, first, a phase-contrast and then an electron microscope. After the termination of stirring the PRP was drawn off and the wells were washed with Tyrode solution (137 mM NaCl, 2.7 mM KCl, 0.36 mM Na_3PO_4, 0.1% glucose, and 1 mM $MgCl_2$, pH 6.5), in which the number of platelets was counted. Then 2.5% glutaraldehyde in Tyrode solution was added in a volume of 0.5 ml to each well incubated at room temperature during 2 hours and washed with physiological saline. The microscopic picture of platelet aggregation was observed with a phase-contrast microscope (×600). The well bottoms were then cut off, the samples were dehydrated in ascending concentrations of alcohol (30-100°), shadowed with silver, and examined under an electron microscope (×2500-10,000).

RESULTS

The proposed model of thrombogenesis has the following advantages: 1) reproduction of high shear rates at the well bottom, comparable to the shear rates near the vessel wall in large elastic vessels; under our conditions the shear rate is 500 sec^{-1} at 1 rev/sec stir rotation; 2) the possibility of phase-contrast microscopic examination of the samples directly on the well bottoms; 3) the possibility of studying different stages of platelet-collagen substrate interaction.

The data of the phase-contrast microscopy of platelet-collagen substrate interaction are presented in Fig. 2, *a*.

Platelet aggregation is collagen-dependent.

These preliminary results are confirmed by the number of platelets remaining in the PRP after interaction with the collagen substrate or non-coated bottom. The PRP contained 2.1×10^8 cells/ml in the first and 4.4×10^8 cells/ml in the second case, the initial number of platelets being 5×10^8 cells/ml.

Thus, 2.8×10^8 cells/ml remained after interaction of PRP with the collagen substrate in the form of aggregates attached to the well bottom.

For a more detailed study of the platelet-collagen interaction an additional experimental series was undertaken with the use of an electron microscope. The results are presented in Table 1.

As seen from the Table, platelet aggregation is collagen-dependent, since no signs of platelet activation were seen after interaction of PRP with non-coated plastic (Fig. 3, *a*). Monomolecular collagen induces contact activation and aggregation of platelets. All stages of platelet activation can be observed: formation of pseudopodia (Fig. 3, *b*), adhesion (Fig. 3, *c*), plating on the collagen surface (Fig. 3, *d*), adhesion of platelets from the suspension to the surface of a plated platelet (Fig. 3, *e*), formation of a platelet clot (Fig. 3, *f*), organization of the clot with fibrin threads (Fig. 3, *g*) after 60 min stirring, and clot retraction (Fig. 3, *h*).

The proposed system is the simplest model simulating *in vivo* thrombogenesis. The system allows for step-by-step observation of the platelet-collagen interaction at high shear rates comparable to those near the walls of large arteries, as well as of the effect of inhibitors and activators of thrombogenesis.

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