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Research Article

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ABSTRACT Intravascular coagulation, thrombosis, and fibrin deposition often produce tissue damage in allogeneic inflammatory reactions such as allograft rejection. The mechanisms which initiate blood clotting in these reactions are poorly understood. We find that allogeneic stimulation of human leukocytes in vitro increases production and expression of tissue thromboplastin-like activity. In our experiments mixed leukocyte cultures (MLC) of cells from allogeneic (unrelated) donors produced and expressed more procoagulant activity than control cultures of cells from each donor alone. After 7 days, allogeneic MLC had 5- to 50-fold more total procoagulant activity than controls, as shown by assaying lysed whole cultures. Additionally, allogeneic MLC had 8- to 240-fold more procoagulant activity expressed on leukocyte surfaces and in culture supernates than controls after 7 days, as shown by assaying intact whole cultures and cell-free supernates. These increases were largely accounted for by gains in the amounts of procoagulant activity produced and expressed per cell in MLC as compared to controls. Controls and MLC produced and expressed considerable amounts of procoagulant activity during the 1st day of culture, and there were no differential effects of allogeneic stimulation on day 1. However, after day 1, the total amount of procoagulant activity produced and the amount expressed declined steadily in controls, nearly reaching preculture levels by day 7. In contrast, the total amount of procoagulant activity in allogeneic MLC remained high, and the amount of activity expressed on cell surfaces and in supernates increased severalfold by day 7. MLC of syngeneic (identical twin) cells produced and expressed the same amount of activity as controls over a 7-day period, whereas MLC of cells from each twin and an allogeneic

donor produced and expressed more activity than controls (at least 9- and 35-fold more, respectively). Thus, increases of procoagulant activity production and expression were found only in MLC of genetically dissimilar cells. Therefore, these increases must have resulted from allogeneic stimulation.

INTRODUCTION

Tissue thromboplastin is a procoagulant lipoprotein which activates Factor VII(2) and thereby initiates coagulation through the extrinsic clotting system. Mechanisms responsible for stimulating cellular production of tissue thromboplastin activity may be of considerable importance in producing thrombosis and inflammation. Tissue thromboplastin apoprotein has been demonstrated on the surfaces of a variety of cell types by an immunoperoxidase technique (3). Lipoproteins with tissue thromboplastin activity can be extracted from organs such as lung, placenta, and brain, but only very small amounts of this activity have been demonstrated in freshly isolated cells.

Leukocytes participate in inflammatory reactions and there is evidence indicating that these cells can activate coagulation proteins. Freshly isolated normal human leukocytes have little or no procoagulant activity (4). However, leukocytes incubated in the presence of plasma, serum, endotoxin, phytohemagglutinin, or purified protein derivative are stimulated to produce large amounts of tissue thromboplastin-like activity (5-9). Leukocytes with this activity are capable of initiating disseminated intravascular coagulation, thrombosis, and Shwartzman-like reactions when infused into experimental animals. In addition, endotoxin injections have been found to stimulate in vivo production of procoagulant activity by leukocytes in animals undergoing classical Shwartzman reactions (10, 11).

Experimental and clinical evidence indicates that immunologic diseases such as lupus erythematosus,

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rheumatoid arthritis, and allograft rejection resemble the classical Shwartzman reaction in that considerable tissue damage may be produced by thrombosis and (or) fibrin deposition (12–24). However, very little is known about mechanisms which initiate blood coagulation in these immunologic diseases (25). Recently, we have shown that incubation of human leukocytes with IgG of at least three of the four subclasses enhances *de novo* production of a tissue thromboplastin-like activity by these cells, and soluble antigen-antibody complexes are especially effective (4). In the present study, we show that *in vitro* allogeneic stimulation of human leukocytes increases production and expression of tissue thromboplastin-like procoagulant activity.

METHODS

Isolation and culture of leukocytes. Human venous blood was collected in evacuated 10-ml glass tubes (Becton, Dickinson & Co., Rutherford, N. J.) containing 15 mg of potassium EDTA or in a sterile syringe containing heparin (2 U/ml). These two methods gave similar results. Leukocytes were isolated by centrifugation of washed buffy coats on Ficoll-Hypaque (Ficoll, Sigma Chemical Co., St. Louis, Mo.; Hypaque, Winthrop Laboratories, New York) gradients as previously described (4). The resultant leukocyte population contained a mean of 90% lymphocytes and 10% monocytes as well as small numbers of erythrocytes and platelets.

Isolated leukocytes were cultured in 5-ml polypropylene tubes using RPMI-1640 tissue culture medium containing 25 mM Hepes buffer, 17% human AB serum, and 2 mM L-glutamine. Serum was prepared from whole blood clotted for 4 h at 37°C. Serum was then heated at 56°C for 45 min to inactivate complement and thrombin. In some experiments serum was also absorbed three to four times with aluminum hydroxide as described elsewhere (26) to remove Factor VII. Absorbed serum was free of detectable Factor VII (<0.1% activity) as shown by a one-stage assay using Simplastin (Ortho Diagnostics, Raritan, N. J.) in Factor VII-deficient plasma.

Mixed leukocyte cultures (MLC)¹ were started with a concentration of 1×10^6 leukocytes/ml from two donors (giving a total leukocyte concentration of 2×10^6 leukocytes/ml). Unmixed control cultures were started with 2×10^6 cells/ml from each donor individually. These controls gave results similar to other controls with 1×10^6 leukocytes/ml. Four to six replicate cultures were carried out for every condition.

Cultures were incubated for 1–7 days at 37°C in a humidified atmosphere of 95% air and 5% CO₂. At the end of culture, leukocyte buttons were gently suspended by tapping the sides of culture tubes, and replicate cultures were pooled. These pooled preparations were then assayed for procoagulant activity by one-stage tests as described below. To measure total procoagulant activity, cultures were lysed by freezing (–70°C) and thawing (37°C). In some experiments supernates were carefully removed from cultures with a Pasteur pipette without disturbing cell buttons to measure the amounts of procoagulant activity released into the culture medium. Microscope examination of wet mounts prepared from these supernates indicated that intact cells were not present.

Leukocyte counts were determined with a Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.) or with a hemocytom-

eter. Leukocyte viabilities were assayed by the method of trypan blue exclusion (27).

Sterile techniques. Sterile reagents and plasticware were used in all experiments. Complete culture medium was sterilized by filtration through 0.22- μ M filters (Millipore Corp., Bedford, Mass.) immediately before use. Pipetting procedures were carried out in a sterile tissue culture hood. After culture, leukocyte preparations were examined microscopically to facilitate detection of possible contamination with bacteria and fungi. Contamination was very rare, and only results with uncontaminated preparations are reported. As an additional check for sterility, cultured cells were transferred to tubes containing trypticase soybroth and incubated for 72 h. Bacterial growth was not seen in these tubes.

One-stage assay of procoagulant activity. 0.1 ml of leukocyte culture was placed into a 12 \times 75-mm glass tube in a 37°C water bath. 0.1 ml of 0.025 M CaCl₂, followed by 0.1 ml of citrated human plasma was added, and the clotting times were then determined. Substrate plasma was obtained from normal human subjects and from patients congenitally deficient in Factor VIII or Factor IX. Congenitally deficient plasmas with <1% of normal Factor V, VII, or X activity were obtained commercially (George-King Biomedical, Inc., Salem, N. H.). Three different Factor VII-deficient plasmas (lots GK 702-629; Gk 703-714; GK 701-522) gave similar results in our experiments.

Conversion of clotting time to relative procoagulant activity: calculation of thromboplastin units. The amount of procoagulant activity present in each MLC was compared with the amounts in controls from the same experiment. (Controls contained cells from the two donors cultured individually.) This comparison was made by first converting clotting times to percent activity using a calibration curve and then to relative procoagulant activity, as described below.

Calibration curves were prepared by assaying the procoagulant activity of doubling dilutions of MLC lysed by freeze-thawing on day 6 or 7. Dilution was plotted against clotting time on log-log paper. The clotting time obtained with the undiluted preparation was assigned a procoagulant activity value of 100%, a 1:1 dilution a value of 50% and so on. Analysis of 18 MLC harvested on days 6 and 7 showed a logarithmic relationship between clotting time and procoagulant activity throughout the range of clotting times encountered (48–300 s). Individual curves were derived for most experiments. In a few experiments the median slope (–0.196) of these calibration curves (range –0.183 to –0.235) was utilized. To determine relative procoagulant activity, ratios of percent activity were calculated, such as percent MLC/percent control I or percent control I/percent control II. (Thus, the control used as the dividend always had a relative procoagulant activity of 1, and the relative activities of other cultures varied.) Values of relative procoagulant activity represented different clotting times in each experiment since cell preparations, substrate plasmas and calibration curves differed from one experiment to another. In some experiments relative activities were normalized for leukocyte concentration. This was done by dividing the activity values obtained from calibration curves by the concentration of leukocytes present in culture at the time of harvest.

The amounts of rabbit brain tissue thromboplastic (Difco Laboratories, Detroit, Mich., generously donated by Dr. Julian Niemetz) needed to give the same clotting time as cultured cells were determined by performing one-stage assays of doubling dilutions of brain thromboplastin suspended in culture medium and plotting a calibration curve. A logarithmic relationship between brain thromboplastin dilution and clotting time was found (slope –0.05). 0.1 ml of the undiluted preparation was defined as 100 units of activity, a 1:10 dilution

¹ Abbreviations used in this paper: MLC, mixed leukocyte culture; RPMI-1640, a synthetic tissue culture medium.

as 10 units, a 1:100 dilution as 1 unit, and so on. (5 units gave a clotting time of 54 s, 2.5 units a time of 71 s, 1.25 units a time of 92 s, 0.625 units a time of 113 s, and 0.317 units a time of 134s.) Brain thromboplastin units corresponding to clotting times of MLC and control leukocyte cultures were read off the curve. Relative procoagulant activities (MLC: control) were not identical to ratios of MLC: control thromboplastin units since the slopes of calibration curves derived with leukocytes and brain thromboplastin were different (see above). The assay must be considered semiquantitative because of this difference in slopes and because the material assayed is crude.

RESULTS

Demonstration that increased amounts of procoagulant activity are expressed on cell surfaces and in supernates of MLC. In order to measure the amounts of procoagulant activity of cultured leukocytes that might be considered as physiologically "expressed", we assayed intact whole cultures containing viable cells. This measurement included procoagulant activity bound to leukocyte surfaces and activity released into supernatants. Leukocytes did not express measurable procoagulant activity before incubation, (i.e., the amount of activity present was the same as with culture medium alone). However, after one day of culture considerable amounts of activity were expressed in MLC as well as in individual donor controls, as indicated by results on day 1 (Fig. 1A). During the next few days, the amount of expressed activity diminished steadily in controls. In contrast, expressed activity in MLC remained at high levels and increased an additional severalfold by day 4-7. After this increase, MLC consistently showed greater amounts of expressed activity than controls harvested even at their peak activity (day 1).

To measure the amounts of expressed procoagulant activity released from cells, cell-free culture supernates were assayed. Very little activity was detected in the supernates of controls throughout the 7 days of culture. Although very little activity was found in the supernates of MLC before day 3, supernatant activity increased considerably in MLC after 3-7 days. Consequently, after 7 days of culture, supernates from MLC showed greater procoagulant activity than controls. However, the amounts of activity found in supernates of MLC were small relative to the total amount of expressed activity in whole intact cultures (Table I). Since the difference between the amount of total expressed activity and the amount in the supernate represented leukocyte surface activity, expressed activity was mostly surface bound.

Demonstration that increased amounts of procoagulant activity are present intracellularly in MLC. To measure intracellular procoagulant activity we compared results with whole cultures assayed intact (unlysed) and cultures which were lysed at the time of harvest. Assays of lysed cultures gave the total amounts of procoagulant activity produced (Fig. 1B). The in-

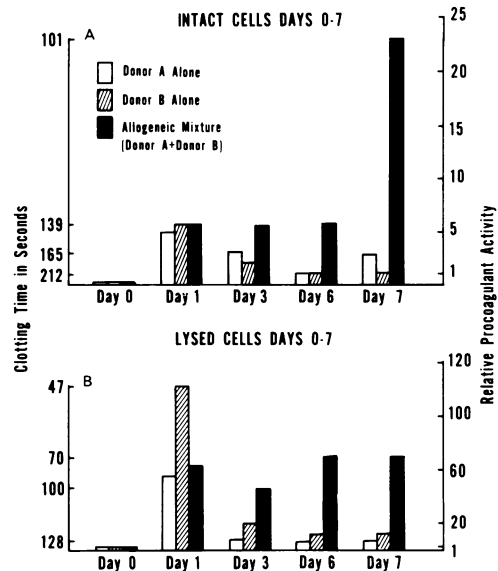


FIGURE 1 The production and expression of procoagulant activity in MLC of leukocytes from two allogeneic donors are compared with controls containing each donor alone. The total amounts of activity produced, including intracellular, cell surface, and supernatant activities are indicated by results with lysed cells (B). Expressed activity, including only cell surface and supernatant activities, is indicated by results with intact cells (A). (Note: In this experiment the intact control with the smaller amount of procoagulant activity on day 7 was assigned a relative activity of 1, and the relative activities of MLC and other controls varied. The scales used in the A and B are different due to the large increase in activity after lysis of cells.)

creased activity seen with lysed as compared to intact preparations was considered to represent intracellular activity. Leukocytes did not show any measurable intracellular procoagulant activity before incubation. After the 1st day of culture, large amounts of intracellular activity were found in MLC and controls, as indicated by results on day 1 (Fig. 1). Occasionally, the intracellular procoagulant activities of MLC and controls were significantly different on day 1, but these differences were relatively small (median <2.5-fold); either MLC or control preparations gave greater activity in various experiments. After day 1, intracellular

TABLE I
Relative Procoagulant Activities of Cell-Free Culture Supernates and Intact Cultures on Day 7

	Intact cultures	Cell-free culture supernates
MLC	71 (96 s)*	11 (140 s)
Donor I (control)	4.6 (165 s)	2.5 (186 s)
Donor II (control)	2.7 (183 s)	1.0 (220 s)

* Numbers within parentheses indicate clotting times.

TABLE II
Relative Procoagulant Activities of MLC on Days 1-7

	Day 1		Day 3		Day 6		Day 7	
	Hi*	Lo*	Hi	Lo	Hi	Lo	Hi	Lo
Intact cells								
Median	1.4	2.5	2.9	5	20	32	29	53
Range	0.67-3.5	1-10.1	0.36-18	1.2-105	5.7-224	5.7-330	8.3-37	13-241
Lysed cells								
Median	0.88	1.7	2.2	5.4	4.5	11.2	8.4	24
Range	0.41-8.7	0.86-12.6	0.93-8.1	1.4-11	1.3-50	4.2-166	5.3-15	11-50
Number of allogeneic combinations tested	15		19		19		9	

MLC and individually cultured controls were harvested after 1-7 days. Results compiled from a number of experiments using a panel of allogeneic leukocyte donors are shown. The procoagulant activity of each MLC was compared to the two individually cultured controls from the same experiment and day. Ratios of MLC/control activity, i.e., "relative procoagulant activities" of MLC, were calculated as described in Methods.

* Often the two cultures for each MLC produced slightly different amounts of activity. Therefore, MLC activity was calculated relative to both controls. "Hi" designates the MLC/control ratio using the control producing the greater amount of procoagulant activity. "Lo" is the MLC/control ratio using the control producing the lesser amount.

activity in controls diminished steadily, nearly reaching preculture levels within 4-7 days. In contrast, intracellular activity in MLC was sustained at high levels throughout the 7-day period of observation. Consequently, MLC had greater amounts of intracellular activity than controls by day 7.

Range of procoagulant activity increases in MLC. To examine the range of procoagulant activity increases in response to allogeneic stimulation, cells from 16 unrelated donors were tested in MLC. To assure a broad survey, cells from each of these donors were used in two allogeneic combinations at most. After only 1 day of culture there was no consistent difference in the amounts of expressed activity in MLC and controls (Table II, intact cells). After 6 days of culture, all 19 intact MLC expressed much more procoagulant activity than controls, and by day 7 these differences were even more pronounced. Similarly, lysed MLC showed the same amounts of activity as controls on day 1, indicating that the total activities (including intracellular and expressed) were the same in these preparations. However, by day 7 lysed MLC showed greater amounts of procoagulant activity than controls in all of the nine combinations tested (Table II, lysed cells). Thus, the amounts of expressed and total procoagulant activity were significantly greater in MLC than controls by day 7 in every allogeneic combination tested.

Demonstration that procoagulant activity production is stimulated by allogeneic interactions: absence of stimulation with identical twins. To demonstrate that increases of procoagulant activity in MLC result from allogeneic stimulation rather than from other factors, the amounts of activity produced and expressed in MLC of cells from syngeneic (identical) twins were

compared with the amounts in controls and allogeneic (unrelated donor) MLC. Syngeneic MLC were found to have the same amounts of procoagulant activity as individually cultured controls on days 1-7. In contrast, allogeneic MLC containing leukocytes from either twin and an unrelated donor had far greater amounts of activity than controls by day 7 (Fig. 2). Similar observations were made with three sets of identical

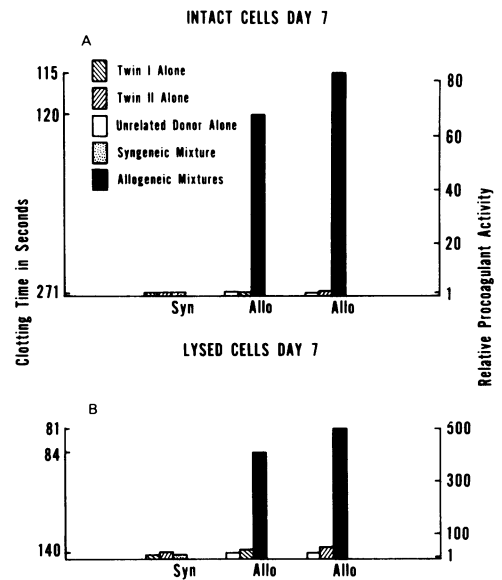


FIGURE 2 The amounts of procoagulant activity produced and expressed by MLC containing syngeneic cells (twin I and twin II) or allogeneic cells (twin II and unrelated donor) are compared. Relative procoagulant activity was calculated as in Fig. 1.

twins. Since increases of activity were only seen in MLC of genetically dissimilar cells, nonspecific cellular interactions and effects of possible contaminants such as endotoxin cannot account for the increases observed. Rather, allogeneic stimulation must have triggered these increases.

Demonstration that increased amounts of procoagulant activity are produced and expressed per cell in allogeneic MLC. To determine the amounts of procoagulant activity produced and expressed per cell in MLC and controls, leukocyte counts and viabilities in culture were monitored throughout the period of observation. Viability was found to be the same in all preparations through day 7 (99–100%). However, somewhat higher leukocyte concentrations were found in MLC than in controls by day 5–7 (median: 1.25-fold higher on day 7). Differences of leukocyte concentration were quite small relative to differences of procoagulant activity. Consequently, there was much more procoagulant activity produced and expressed per cell in MLC than in controls (Table III). An additional demonstration of this effect was provided by a study of MLC started with 50% fewer cells than controls. After 7 days of culture, leukocyte concentrations in these MLC did not exceed controls, but the amounts of intracellular and expressed procoagulant activity were much greater than in controls. Thus, in MLC the amount of activity per cell was greater than in controls.

Demonstration that the procoagulant produced by MLC is tissue thromboplastin-like. The procoagulant activity produced in MLC was found to have tissue thromboplastin-like properties. To characterize this activity cultures were carried out in RPMI-1640 medium containing absorbed serum free of detectable Factor VII activity. After 7 days, the cultured leukocytes were washed in RPMI-1640 medium (without serum) and assayed in plasmas deficient in single clotting factors. These leukocytes shortened clotting times in Factors VIII- and IX-deficient plasmas to the same ex-

TABLE III
Relative Procoagulant Activities of MLC on Day 7. Results Normalized for Leukocyte Concentration at the Time of Harvest

	Hi*	Lo*
Intact cells		
Median	19	27
Range	5.6–23	8.7–106
Lysed cells		
Median	8	18
Range	3–12	7.6–28
Number of allogeneic combinations tested		9

* See legend, Table II.

TABLE IV
The Tissue Thromboplastin-Like Procoagulant Produced in MLC Requires Factor VII

	Clotting time in normal and deficient plasmas			
	Normal plasma	VII Def.	VIII Def.	IX Def.
	s			
Washed MLC cells	87	175	90	100
Medium alone	310	165	>360	>360

tent as in normal plasma (Table IV) indicating that the activity produced in MLC does not act through Factors VIII and IX or the intrinsic pathway of coagulation. On the other hand, clotting times in Factor VII-deficient plasmas were not shortened by these leukocytes, indicating that the activity produced is like tissue thromboplastin in that it requires Factor VII. The amounts of a crude rabbit brain thromboplastin needed to give the same clotting times as MLC and controls were calculated to show how much tissue thromboplastin-like activity was expressed in these cultures relative to an available standard (Table V). The relative increase in activity of MLC vs. control was not as great when the calibration curve was constructed with the tissue factor standard as when the MLC was used for this purpose. This difference is the result of the different slopes obtained with the two materials. The difference in slopes suggests that human leukocyte procoagulant activity differs from rabbit tissue factor in some way, or that other leukocyte constituents effect the results. Leukocytes from MLC did not shorten clotting times in Factors V- and X-deficient plasmas.

DISCUSSION

The experiments reported here demonstrate that allogeneic stimulation increases the production and ex-

TABLE V
Amounts of Standard Thromboplastin Activity Expressed by MLC and Control Cultures

	Clotting time	Relative procoagulant activity	Brain thromboplastin units
	s		
MLC	75	26	2.0
Donor I	134	1.3	0.4
Donor II	141	1.0	0.3

The amounts of procoagulant activity expressed by intact MLC and control cultures as measured by a one-stage assay are shown as clotting times, relative procoagulant activities, and number of standard thromboplastin units.

pression of a tissue thromboplastin-like procoagulant activity by human leukocytes. In these experiments allogeneic leukocytes in MLC were found to produce and express greater amounts of procoagulant activity than syngeneic leukocytes in MLC or controls with each donor alone. These findings were shown to be largely due to increases in the amount of procoagulant activity produced and expressed per cell after allogeneic stimulation.

Experiments with identical twins showed that our findings were due to allogeneic stimulation rather than nongenetically determined factors. MLC containing syngeneic leukocytes (cells from twins) were found to produce and express no more activity than controls with each twin alone. On the other hand, MLC containing cells from either twin in allogeneic combination (with cells from an unrelated donor produced and expressed more activity than controls. Similar results were obtained in experiments using three sets of identical twins. Thus, increased procoagulant activity production and expression only occurred in MLC containing phenotypically dissimilar cells and the importance of allogeneic stimulation in these experiments was demonstrated.

Although isolated leukocytes had no measurable procoagulant activity before culture, activity was found in controls after culture for 1 day. Most of this activity was contained intracellularly, but some was also expressed on cell surfaces. There were no consistent differences between MLC and controls on day 1. (The appearance of this activity on day 1 may be attributed to stimulatory effects of serum factors in the culture medium, since cells cultured for this period in synthetic medium without serum fail to generate significant amounts of procoagulant activity) (4). After day 1 activity in control cultures diminished steadily. In contrast, in MLC, intracellular activity was sustained throughout the 7-day period of observation. Furthermore, expressed activity increased an additional severalfold between days 4 and 7, reaching levels greater than in controls even at peak activity (day 1). This increase was mostly due to augmentation of cell surface activity, but some additional activity was also released into the supernates. By day 7, the amounts of intracellular, cell surface, and supernatant activities were all greater in the MLC than in controls.

Other investigators have recently reported that they were unable to find increases in procoagulant activity in MLC assayed after 1, 2, and 3 days of culture (28). Our findings are in agreement with these workers in that the increases in procoagulant activity of MLC over controls were not seen after short-term culture. However, our experiments show clearly that after 7 days of culture, activity is very significantly increased in MLC.

These findings may be of importance in several situations. The MLC has been widely used as an in vitro

model of histocompatibility and as a tool for defining leukocyte histocompatibility antigens. The biologic response normally assayed has been one of cell growth measured as [³H]thymidine incorporation into cultured cells (29). However, lymphokine production assayed as lymphocytotoxic or migration inhibitory factor production have also been shown to be useful in this regard (30, 31). Our findings indicate that production and expression of tissue thromboplastin-like activity is yet another part of the MLC reaction and this activity may play a role in the pathophysiology of allograft rejection (see below). It is therefore possible that measurement of tissue thromboplastin-like activity in allogeneic leukocyte cultures will prove to be of value in studying histocompatibility.

Intravascular coagulation and/or thrombosis appear to be pathogenetic in allogeneic tissue reactions such as allograft rejection, graft-vs.-host and host-vs.-graft phenomena (18, 24, 32, 33). The mechanisms which initiate clotting in these reactions are poorly understood. Tissue thromboplastin is a potent initiator of the coagulation sequence and it is reasonable to propose that production of tissue thromboplastin by allogeneically stimulated leukocytes may contribute to tissue damage in these inflammatory reactions.

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