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LYMPHOKINES AND THROMBOSIS

II. Procoagulant Activity Produced by Human Lymphocytes Stimulated with Concanavalin A (Con-A)

By

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Recent studies have demonstrated that fibrin accumulation and thrombosis are regular features of cell-mediated immune reactions (8). The inflammatory changes of cell-mediated type IV immune reactions are initiated by biologically active compounds, lymphokines, that are released from lymphocytes, which react specifically with antigen (1). Lymphokines are also released from lymphocytes that are stimulated with mitogens, as for instance, concanavalin-A (Con-A). Lymphokines are multipotent biological products, which can be assumed to interfere with blood coagulation and thrombosis in several ways, e.g. vessel wall damage due to cytotoxic activity directly on endothelial cells, or mediated through lymphokine activated and chemotactically attracted granulocytes or monocytes. Thrombocyte aggregating activity (TAA) of lymphokines has recently been demonstrated (7, 13) and represents another potentially important mechanism by which lymphocyte factors may initiate thrombotic processes in type IV reactions. Lavelle et al. described in an animal model, specific, antigen-induced lymphocyte production of TAA, but did not find any procoagulant activity (13). The present study extends research on the association between lymphokines and thrombosis by investigating whether products released from human lymphocytes contain substances which influence blood coagulation.

MATERIAL AND METHODS

Lymphokine Production

Con-A-released human lymphokines were produced by *in vitro* incubation of isolated human peripheral blood lymphocytes, and removal of Con-A by passage through a Sephadex® G-100 column as indicated by Bendtzen et al. (3) and Coeugniet et al. (5, 6). The Con-A stimulated and control culture supernatants were examined for leucocyte migration inhibitory activity (LMIA) by means of the leucocyte migration agarose test (LMAT) as described by Clausen (4) with minor modifications, and the material was concentrated by lyophilization. Six pools, each containing equal quantities of active (AS) and control (CS) supernatants from six persons were prepared. The LMIA of the lyophilized pools of supernatants was tested by the LMAT after redissolution in TC 199 in order to ensure that the biological activity of the samples had been preserved (Table 1). All these operations have previously been described (7).

The effect on blood coagulation was examined with AS and CS redissolved in 0.1 M Tris buffer HCl, pH 7.35 to give a final concentration equal to the concentration of the initial culture supernatant before Sephadex passage (IC), three times more concentrated ($3 \times \text{IC}$) and three times diluted ($\text{IC}/3$).

Tests for Effect of Culture Supernatants on Blood Coagulation

The influence of AS, CS and a control with buffer only (CB) was studied on:

1. recalcification time of thrombocyte rich plasma (TRP) and thrombocyte poor plasma (TPP).
2. partial thromboplastin time.
3. thrombin clotting time of TRP and TPP.
4. thrombin clotting time of human fibrinogen solution.
5. direct action of supernatants on human fibrinogen solutions.
6. spectrophotometric evaluation of plasma fibrinogen polymerization.

1. Recalcification time of TRP and TPP: TRP and TPP were prepared as previously described (7). An amount of 0.1 ml AS, CS or CB was added to 0.1 ml citrated TRP or TPP. The mixture was heated in glass tubes at 37° C in water bath for 60 sec, 0.1 ml 0.025 M CaCl_2 was added, and the time taken for the clot to appear was recorded with a stop-watch.

2. Partial thromboplastin time: 0.1 ml TPP was mixed with 0.1 ml AS, CS or CB and with 0.1 ml partial thromboplastin time reagent (Pathromtin®, Behringwerke, Germany) and incubated for 60 sec at 37° C. An amount of 0.1 ml 0.025 M CaCl_2 was added and the time taken for the clot to appear was recorded with a stop-watch.

3. Thrombin clotting time of TRP and TPP: 0.1 ml TRP and TPP was mixed with AS, CS or CB and incubated at 37° C for 60 sec, whereupon 0.1 ml of a human thrombin solution (5 NIH/ml) was added. The time from the

moment when the thrombin was introduced into the system until the clot appeared was recorded.

4. *Thrombin clotting time of human fibrinogen solution:* Twenty milligrammes human fibrinogen (human lyophilized fibrinogen, Grade L, Kabi, Sweden) was dissolved in 8 ml 0.1 M Tris buffer pH 7.35 and left for 30 min at 35° C. Two millilitres 0.025 M CaCl₂ was added and 0.1 ml of the final solution was mixed with AS, CS or CB and incubated at 37° C for 60 sec, whereupon 0.1 ml of a human thrombin solution (5 NIH/ml) was added. The time from the moment when the thrombin was introduced into the system and until the clot appeared was recorded.

5. *Direct action of culture supernatants on human fibrinogen solution:* The human fibrinogen solution was prepared as described above except that the fibrinogen was dissolved in Tris buffer containing final concentrations of AS and CS in $3 \times \text{IC}$, IC or IC/3. One millilitre of the supernatant-fibrinogen solution was placed in a glass tube at 37° C. After 15, 30, 60 and 120 min the tube was examined and the coagulation registered.

6. *Spectrophotometric evaluation of plasma fibrinogen polymerization:* The influence of AS and CS in $3 \times \text{IC}$, IC and IC/3 on plasma fibrinogen polymerization was followed, using the spectrophotometric method described by Ferry & Morrison (9). An amount of 200 μl TPP and 120 μl AS or CS was added to 2 ml Tris buffer HCl, 0.1 M, pH 7.35. The samples were left in cuvettes for 5 min at room temperature and recalcified with 0.2 ml 0.025 M CaCl₂. After recalcification the optical density was recorded for each sample in a Beckman spectrophotometer at 350 μm every 20 sec until a plateau was obtained. The interval from introduction of CaCl₂ until the beginning of polymerization (lag period) and the duration of polymerization was recorded.

TABLE 1

Leucocyte Migration Inhibitory Activity (LMIA) of Culture Supernatants Prepared by Con-A Stimulation of Human Lymphocytes.

Pool no.	Concentration of supernatants		
	$3 \times \text{IC}$ MI $\pm$ S.D.	IC MI $\pm$ S.D.	IC/3 MI $\pm$ S.D.
1	0.36 $\pm$ 0.10	0.59 $\pm$ 0.05	0.71 $\pm$ 0.04
2	0.39 $\pm$ 0.10	0.58 $\pm$ 0.07	0.73 $\pm$ 0.05
3	0.41 $\pm$ 0.10	0.63 $\pm$ 0.03	0.73 $\pm$ 0.05
4	0.41 $\pm$ 0.10	0.61 $\pm$ 0.05	0.71 $\pm$ 0.00
5	0.36 $\pm$ 0.06	0.49 $\pm$ 0.03	0.68 $\pm$ 0.06
6	0.38 $\pm$ 0.08	0.59 $\pm$ 0.08	0.71 $\pm$ 0.03

IC: Initial concentration. MI: Migration index. S.D.: Standard deviation.

TABLE 2

Modification by Culture Supernatants of Human Lymphocytes of TRP and TPP Recalcification Time, of Partial Thromboplastin Time, of TRP and TPP Thrombin Clotting Time and of Thrombin Clotting Time of Human Fibrinogen.

Test	Concentration of supernatants					
	3 × IC		IC		IC/3	
	Type of supernatant		Type of supernatant		Type of supernatant	
	AS	CS	AS	CS	AS	CS
	mean ± S.D. clotting time sec (% from CS) (<i>P</i> <)	mean ± S.D. clotting time sec (% from CS) (<i>P</i> <)	mean ± S.D. clotting time sec (% from CS) (<i>P</i> <)	mean ± S.D. clotting time sec (% from CS) (<i>P</i> <)	mean ± S.D. clotting time sec (% from CS) (<i>P</i> <)	mean ± S.D. clotting time sec (% from CS) (<i>P</i> <)
Recalcification time of TRP	74.4 ± 18.2 (70) (0.01)	103.1 ± 10.0 (100)	89.4 ± 14.3 (80) (0.01)	108.8 ± 5.8 (100)	104.4 ± 4.2 (99) (n.s.)	105.9 ± 6.7 (100)
Recalcification time of TPP	160.9 ± 7.0 (84) (0.01)	190.3 ± 7.5 (100)	174.6 ± 5.3 (91) (0.01)	190.6 ± 7.9 (100)	183.5 ± 6.4 (97) (n.s.)	188.9 ± 9.3 (100)
Partial thromboplastin time	39.5 ± 1.0 (70) (0.05)	56.3 ± 2.2 (100)	50.3 ± 1.7 (88) (0.05)	57.3 ± 1.7 (100)	56.3 ± 1.5 (100) (n.s.)	56.5 ± 2.4 (100)
Thrombin clotting time of TRP	10.0 ± 1.2 (62) (0.01)	16.1 ± 2.2 (100)	11.9 ± 0.9 (76) (0.01)	15.5 ± 1.9 (100)	14.0 ± 1.3 (90) (n.s.)	15.6 ± 1.4 (100)
Thrombin clotting time of TPP	20.3 ± 1.0 (76) (0.01)	26.5 ± 1.1 (100)	24.1 ± 0.8 (92) (0.01)	26.1 ± 1.0 (100)	25.4 ± 0.9 (96) (n.s.)	26.5 ± 1.1 (100)
Thrombin clotting time of human fibrinogen	11.3 ± 0.9 (70) (0.01)	16.1 ± 1.4 (100)	13.1 ± 1.0 (84) (0.01)	15.6 ± 0.5 (100)	14.5 ± 0.5 (96) (n.s.)	15.1 ± 0.6 (100)

TRP: Thrombocyte rich plasma. TPP: Thrombocyte poor plasma. AS: Active supernatant. CS: Control supernatant. IC: Initial concentration. n.s.: Non significant. *P*: Significance. S.D.: Standard deviation.

Because the clotting times obtained in control preparations with control supernatant (CS) are nearly identical to those obtained with buffer (CB), the latter are not included in the Table.

TABLE 3
Modification by Culture Supernatants of Plasma Fibrinogen Polymerization
(Spectrophotometric Method).

Parameter	Concentration of supernatants					
	$3 \times \text{IC}$	<i>P</i>	IC	<i>P</i>	IC/3	<i>P</i>
Lag period (sec)	AS: 110 ± 11.5	0.05	140 ± 0.0	0.05	180 ± 0.0	n.s.
	CS: 200 ± 0.0		200 ± 0.0		195 ± 10.0	
Duration of polymerization (sec)	AS: 130 ± 11.5	n.s.	190 ± 11.5	n.s.	195 ± 10.0	n.s.
	CS: 170 ± 20.0		190 ± 11.5		195 ± 10.0	

AS: Active supernatant. CS: Control supernatant. IC: Initial concentration.
P: Significance. n.s.: Non significant.

Calculations

Each experiment was made in quadruplicate and repeated at least four times. The recalcification time, partial thromboplastin time and thrombin clotting time were repeated six times. Statistical significance of the results was evaluated by Wilcoxon's test.

RESULTS

Table 1 shows the LMIA of the culture supernatants in $3 \times \text{IC}$, IC and IC/3. Table 2 shows the recalcification times of TRP and TPP, the thrombin clotting times of TRP and TPP, and the thrombin clotting time of human fibrinogen solution. The results are expressed in seconds, and the AS-induced modification of the clotting time is expressed also in per cent difference from CS modification.

All parameters are modified significantly by AS at a final concentration equal to $3 \times \text{IC}$ and IC. The IC/3 concentration is not active in any of the assays. Clotting times of both TRP and TPP are shortened by AS, but the decrease of clotting time in TRP preparations as compared with CS is higher than in TPP preparations. Thus, recalcification time of TRP is decreased to 70 per cent by AS ($3 \times \text{IC}$) and to 84 per cent in TPP. Likewise, thrombocyte clotting time is decreased by AS ($3 \times \text{IC}$) to 62 per cent as compared with

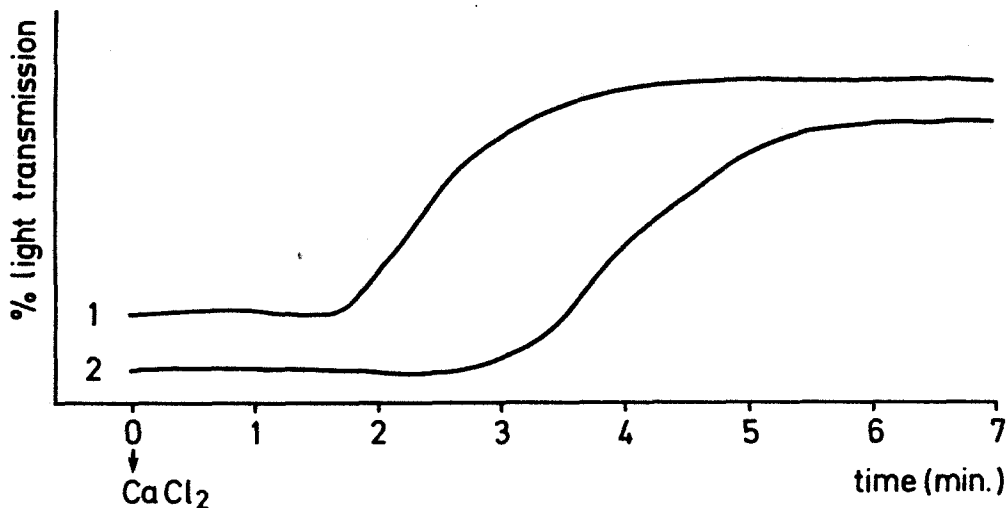


Fig. 1.

Plasma fibrinogen polymerization accelerated by active supernatant (AS) of Con-A stimulated lymphocytes. 1: AS ($3 \times \text{IC}$); 2: CS ($3 \times \text{IC}$). AS: Active supernatant. CS: Control supernatant. IC: Initial concentration.

CS, and to 76 per cent in the TPP preparation. The same effect could be seen with IC supernatants. $3 \times \text{IC}$ and IC of AS also decrease significantly the thrombocyte clotting time of human fibrinogen as well as the lag period of plasma fibrinogen polymerization (Table 3) which is in fact another way of expressing the recalcification time of TPP. None of the culture supernatants modify significantly the duration of

TABLE 4

Direct Clotting Effect of Culture Supernatants on Human Fibrinogen Solutions.

No. tests	Type of supernatants (final concentration)	No. of tubes clotted after:			
		15 min	30 min	60 min	120 min
16	AS ($3 \times \text{IC}$)	3	11	16	16
16	AS (IC)	1	6	8	16
16	AS (IC/3)	0	2	4	5
16	CS ($3 \times \text{IC}$)	0	0	0	0
16	CS (IC)	0	0	0	0
16	CS (IC/3)	0	0	0	0
16	CB	0	0	0	0

AS: Active supernatants. CS: Control supernatants. IC: Initial concentration.

polymerization or the final light transmission (Fig. 1). Table 4 shows that AS exerts a direct concentration and time-dependent coagulating effect on fibrinogen solutions.

DISCUSSION

The study shows that substances produced by human lymphocytes stimulated with Con-A are able to produce a procoagulant activity. In a previous paper (7) we showed that AS contain a proaggregating activity. Since stimulated thrombocytes have by themselves a "procoagulant" effect, the decrease of clotting time in TRP preparations might be due to the same proaggregating activity. In order to illustrate more clearly this mechanism we made parallel experiments using TRP and TPP preparations, and the results support the assumption, since active supernatants containing lymphokines have a higher activity on TRP than on TPP preparations.

The procoagulant activity could be of "thrombin-like" type because of its capacity to decrease the clotting time in test systems with plasma preparations as well as human fibrinogen solutions, and because of a direct clotting effect on human fibrinogen.

The gelation of fibrinogen is thought to be the end result of a reaction sequence composed of at least two steps: proteolysis and polymerization. It is widely believed that thrombin takes part only in the initial proteolytic phase (10) and also other proteolytic enzymes have been described as "clotting enzymes" (2, 10). The "procoagulant" activity may in fact be a proteolytic effect, possibly of lymphokine origin (12).

The existence of a coagulant activity in culture supernatants after lymphocyte stimulation is not surprising since such activities are produced also by other cells (10). The effect could very well be responsible for thrombotic *in vivo* reactions (5, 6, 8). The coagulant activity demonstrated in the present study, and the thrombocyte aggregating effect previously described are both human lymphokine effects that may participate in the pathogenesis of thrombosis during lymphocyte-dependent inflammatory reactions (11, 14, 15).

SUMMARY

Human lymphocytes stimulated with concanavalin-A produce a coagulant activity which decreases the clotting time as expressed through the recalcification time of citrated plasma, the partial thromboplastin time, the thrombin clotting time of citrated plasma and the thrombin clotting time of fibrinogen solutions. The culture supernatants of human lymphocytes stimulated with concanavalin-A also have a direct coagulant effect on human fibrinogen solution. They decrease the lag period of recalcification time of citrated plasma but do not modify the duration of polymerization as measured with a spectrophotometric method.

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