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LYMPHOKINES AND THROMBOSIS

I. Thrombocyte Aggregating Activity Released by Human Lymphocytes Stimulated with Concanavalin-A

By

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Lymphokines are lymphocyte-released factors which act as non-specific mediators of inflammatory damage in cell-mediated (type IV) immune reactions. They are released from T-lymphocytes and B-lymphocytes stimulated by specific antigen or mitogens, as for instance concanavalin-A (Con-A). The lymphokines are compounds with a molecular weight of about 20,000–80,000 daltons and with several biological effects such as leucocyte migration inhibitory activity, macrophage migration inhibitory activity, monocyte and granulocyte chemotactic activity, macrophage activating activity, blastogenic activity, and cytotoxic activity.

In experiments with nephritic rabbits, Lavelle, Ransdell & Trygstad (11) reexposed mononuclear cells to the sensitizing antigen and demonstrated production of a factor that induced thrombocyte aggregation, and it was suggested that cell-derived factors of this type might induce thrombocyte aggregation and intravascular coagulation in certain diseases and thereby be of importance as pathogenic factors. The thrombocyte aggregating factor was similar to the lymphokines as regards the rate of synthesis, molecular weight and thermal stability.

The present study demonstrates the *in vitro* production of thrombocyte aggregating activity (TAA) by Con-A stimulated human mononuclear cells from peripheral blood.

MATERIAL AND METHODS

Production of Lymphokines

Con-A released human lymphokines were produced as indicated by Bendtzen et al. (1). Venous blood was collected in 10 ml polysterole tubes (Nuncclon®, Nunc, Roskilde, Denmark) containing 250 i.u. heparin (Leo, Ballerup, Denmark) and 3 ml Hank's balanced salt solution. Mononuclear cells were separated by centrifuging for 40 min at 220 g in an isopaque-ficoll gradient (Lymphoprep®, Nyegaard & Co., Oslo, Norway). A 95-99 per cent pure mononuclear cell population was obtained which contained only a few per cent monocytes. The cells were collected in a 10 ml polysterole tube, washed three times in Hank's balanced salt solution and suspended in serum-free medium TC 199 with penicillin 67 i.u. per ml and streptomycin 67 µg per ml (Difco Laboratories, Michigan, USA) (TC 199). An amount of 250 µg concanavalin-A (Pharmacia, Uppsala, Sweden) in 50 µl TC 199 was added to 3 ml cell suspension (2.5×10^6 cells per ml), and incubated at 37° C in 2 per cent CO₂ in atmospheric air saturated with water vapour. After 22 h, the culture was terminated by centrifuging for 10 min at 670 g, and 50 µl TC 199 was added to the cell-free supernatant.

In each test, a parallel Con-A reconstituted control supernatant was prepared in the following way: 50 µl TC 199 without Con-A was added to a 3 ml cell suspension, identical with the sample, and the mixture was incubated for 22 h. After centrifugation the cell-free supernatant was reconstituted with 250 µg Con-A in 50 µl TC 199.

Removal of Con-A and TC 199 Compounds from Culture Supernatants

Removal of TC 199 compounds: K 16/20 Sephadex G-50 columns (Pharmacia) (bed volume 20 ml) were equilibrated with ammoniumbicarbonate buffer (20 mMol/l), adjusted to pH 7.0 with acetic acid. Six millilitres cell-free Con-A stimulated and control preincubated culture supernatant (supernatant 1) were applied on the column at room temperature.

The soluble compounds of TC 199, which have by themselves a thrombocyte aggregating activity, were removed by passage through the column, and the procedure involved a 1.5 times dilution of supernatant 1 (supernatant 2).

Removal of Con-A: K 16/10 Sephadex G-100 columns (bed volume 16 ml) were equilibrated with ammoniumbicarbonate buffer (20 mMol/l), adjusted to pH 7.0 with acetic acid. Con-A was removed from supernatant 2 by conjugation to Sephadex by column passage of 3 ml of stimulated and control supernatant, and supernatant 2 was hereby diluted another three times (supernatant 3). The two column passages resulted in dilution of the initial 6 ml supernatant 1 into 9 ml of supernatant 2 (by the Sephadex G-50 passage) and into a final dilution of 27 ml of supernatant 3 (by the Sephadex G-100 passage). The mitogen-free supernatants were passed through Millipore® filters (0.45 µm pore size) and lyophilized.

Supernatants from 24 persons were prepared in this way and were collected in four pools, each containing equal quantities of stimulated and control supernatants from six persons.

The leucocyte migration inhibitory activity (LMIA) of the lyophilized supernatant pools was tested after redissolution in TC 199 at a concentration corresponding to the initial supernatant (IC), three times concentrated ($3 \times \text{IC}$) and three times diluted ($\text{IC}/3$).

Assay for LMIA

The indirect leucocyte migration agarose technique (ILMAT) as described by Clausen (3) was employed with few modifications, using unrelated peripheral blood leucocytes from healthy human donors as migratory cells. Venous blood in 10 ml polysterole tubes containing 250 i.u. heparin and 2 ml 5 per cent dextran 250 in saline was allowed to sediment at 37°C for 1 h. The leucocyte rich plasma was withdrawn, centrifuged at 220 g for 5 min and washed three times at 220 g for 5 min in Hank's balanced salt solution. An amount of 22×10^6 cells was resuspended in 90 μl culture supernatant and incubated in quadruplicates at 37°C for 30 min. 7 μl aliquots of cell suspensions (1.5×10^6 cells) were poured in wells punched out in agarose medium placed on plastic petri dishes. After a migration period of 20 h in 2 per cent CO_2 in atmospheric air, saturated with water vapour at 37°C , the migration areas were studied under a projection microscope and measured by planimetry. A migration index (MI) was calculated from the following formula:

$$\text{MI} = \frac{\text{Mean of leucocyte migration areas in Con-A stimulated supernatants}}{\text{Mean of leucocyte migration areas in parallel control supernatants}}$$

A MI of less than 0.85 indicates presence of LMIA in the Con-A stimulated sample. The action of supernatants on thrombocytes was examined with re-dissolved material in Tris buffer HCl, pH 7.35, reconstituted to give a final concentration of $3 \times \text{IC}$, IC and $\text{IC}/3$.

Assay for Thrombocyte Aggregation

Eighteen millilitres venous blood from normal healthy donors were collected in 2 ml of a 3.8 per cent natrium citrate solution. Thrombocyte rich plasma (TRP) was obtained by centrifugation at $140 \times g$ for 5 min and thrombocyte poor plasma (TPP) by centrifugation at $1,000 \times g$ for 15 min. The thrombocyte aggregation was examined by the method of Born et al. (2) using a Payton Dual Channel Aggregation Module. The direct effect on thrombocyte aggregation of Tris buffer HCl pH 7.35 and of Con-A stimulated supernatants (active supernatants (AS)) and control supernatants (CS) dissolved in Tris buffer HCl, pH 7.35, at concentrations equivalent to $3 \times \text{IC}$, IC and $\text{IC}/3$ was studied. Calibration of the test system was performed by studies on the modifi-

cation of thrombocyte response to adenosin diphosphoric acid (ADP) at final concentrations of $1 \mu\text{mol/l}$ and $2 \mu\text{mol/l}$ and to thrombin at a final concentration of 0.1 and 0.2 NIH/ml. The volumes used in the experiment were: 0.5 ml plasma sample, 50 μl concentrated supernatant solution and/or 50 μl ADP solution. The same experiments were repeated with supernatants previously heated at 56°C for 30 min and at 80°C for 30 min.

Assays for Clot Retraction

The influence of AS and CS ($3 \times \text{IC}$, IC and IC/3) on clot retraction was determined by the thrombocyte rich plasma (TRP) clot retraction technique described by Gaetano et al. (10) and in parallel by the whole clot retraction method presented by Taylor et al. (12).

TRP clot retraction: AS and CS were dissolved ($3 \times \text{IC}$, IC and IC/3) in 0.1 mol/l phosphate buffer pH 7.4. An amount of 1.6 ml TRP was mixed with 0.4 ml phosphate buffer containing AS, CS or buffer only. These mixtures were divided in glass tubes with 0.4 ml in each tube, 0.2 ml CaCl_2 0.025 mMol/l was added to each tube, the tubes were gently agitated for 10 sec and placed in a thermostat at 37°C . The clot retraction was evaluated after 30 min. The clot was gently removed, and the expressed volume of serum was measured by means of a Hamilton microliter syringe. The degree of clot retraction was estimated by relating the expressed volume of serum to the initial volume of the mixture. The mean of four simultaneous determinations was always used, and the significance of the results was calculated by Wilcoxon's test.

Whole blood clot retraction: Whole blood was drawn in plastic tubes and placed at once in icy water. Immediately, at the same temperature, the blood was diluted in 0.1 mol/l phosphate buffer, pH 7.4, each tube containing 1.8 ml buffer plus 0.2 ml blood. This buffer contained AS or CS in final concentrations of $3 \times \text{IC}$, IC and IC/3. The blood was clotted with thrombin 0.1 ml (50 NIH/ml) and left for 30 min at 4°C . The clots were transferred and observed at 37°C for clot retraction after 30 min, 60 min and 120 min using the method described above. Each experiment was repeated four times, each time in quadruplicate, and the significance of results calculated by Wilcoxon's test.

TABLE 1

Leucocyte Migration Inhibitory Activity (LMIA) and Thrombocyte Aggregating Activity (TAA) of Con-A Stimulated Lymphocytes.

Concentration	$3 \times \text{IC}$	IC	IC/3
MI mean $\pm$ S.D.	0.23 ± 0.15	0.37 ± 0.07	0.65 ± 0.07
Thrombocyte aggregation	+	—	—

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

Fig. 1.

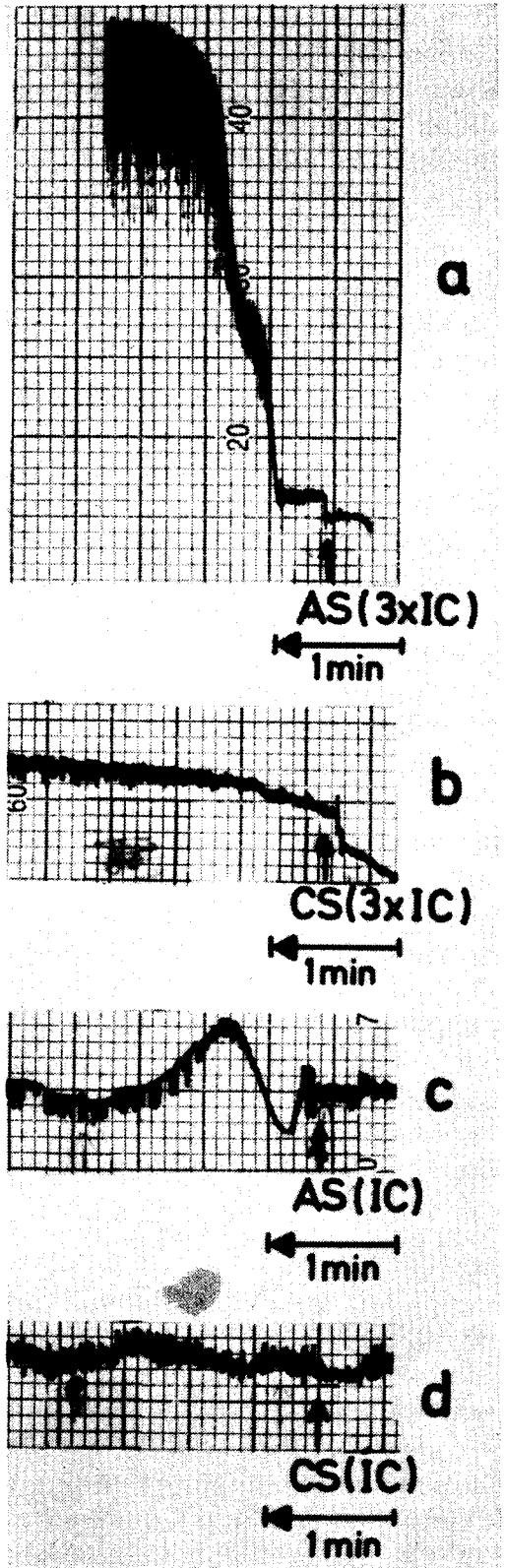
Thrombocyte aggregation induced by lymphokines. Abscissa, time; ordinate, per cent light transmission. Arrow indicates introduction of material. AS, active supernatant; CS, control supernatant; IC, initial concentration.

(a) AS ($3 \times \text{IC}$): aggregation after a latent period of 20 sec.

(b) CS ($3 \times \text{IC}$): no aggregation.

(c) AS (IC): slight and reversible aggregation.

(d) CS (IC): no aggregation.



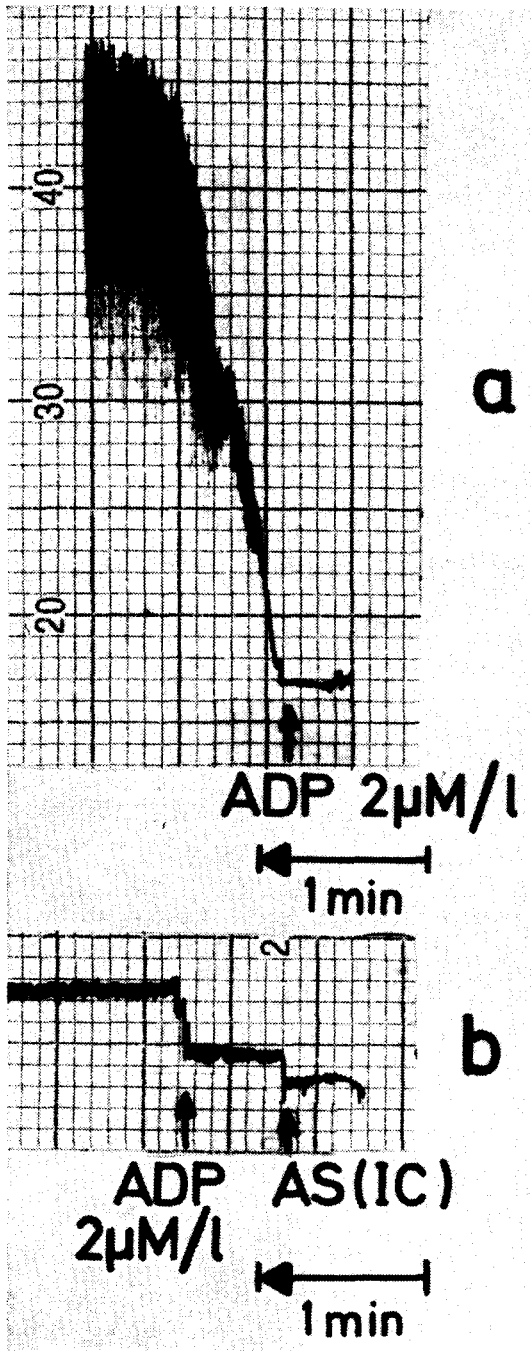


Fig. 2.

Inhibition of thrombocyte response to adenosine diphosphoric acid (ADP) by AS (IC).

Abscissa, time; ordinate, per cent light transmission. Arrow indicates introduction of material.

AS, active supernatant; IC, initial concentration. (a) ADP at a final concentration of 2 μ M/l induces immediate thrombocyte aggregation. (b) A short incubation with AS (IC) (the time space between the two arrows) prevents the aggregating effect of ADP at a final concentration of 2 μ M/l (second arrow).

RESULTS

Table I shows that the supernatants in $3 \times$ IC, IC and IC/3 contained LMIA activity. The concentrations indicated were the final concentrations in the LMIA test system employed, and the reactions clearly showed a concentration dependent

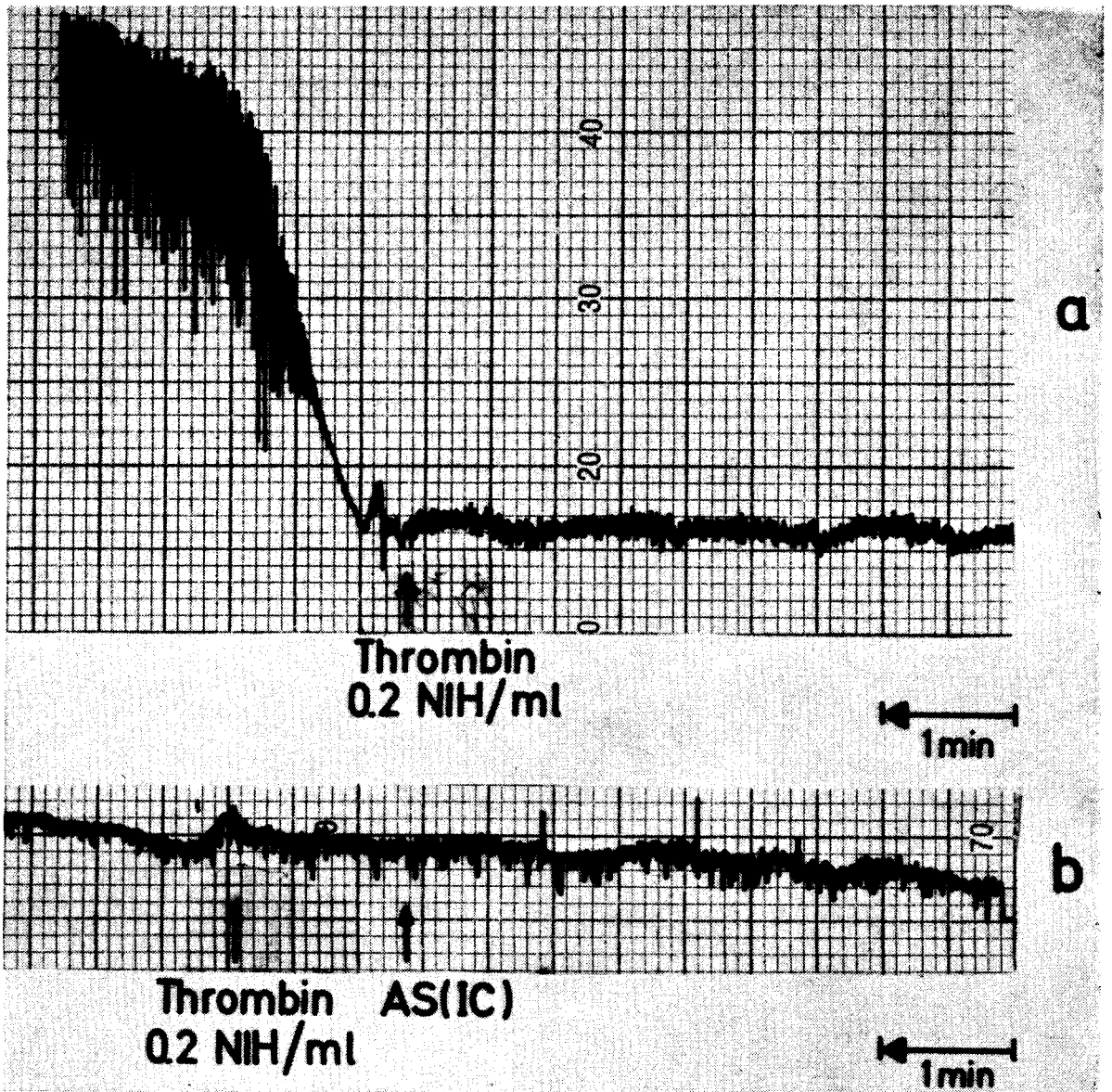
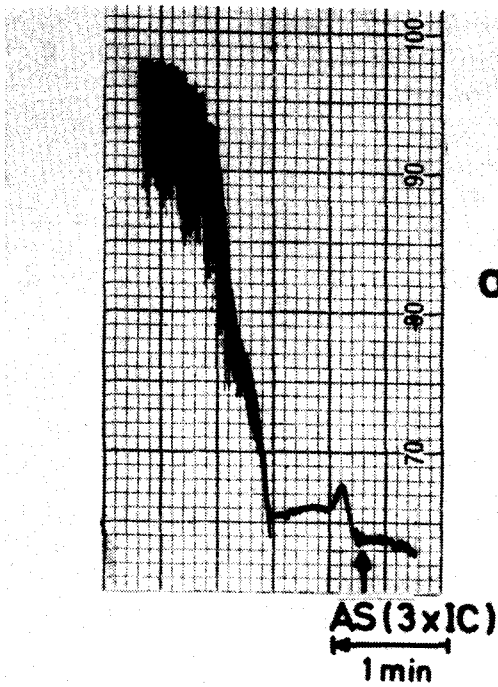


Fig. 3.

Inhibition of thrombocyte response to thrombin by AS (IC). Abscissa, time; ordinate, per cent light transmission. Arrow indicates introduction of material. AS, active supernatant; IC, initial concentration. The aggregating effect of thrombin at a final concentration of 0.2 NIH/ml (a) is prevented by preincubation (space between the two arrows) of thrombocytes with AS (IC).

dose response. The AS pools contained a factor which produced irreversible thrombocyte aggregation at the final concentrations of $3 \times \text{IC}$ (Fig. 1 a). The $3 \times \text{IC}$ CS (Fig. 1 b) did not produce aggregation and the buffer did not modify

**a***Fig. 4.*

Heat inactivation of thrombocyte aggregating activity of AS ($3 \times \text{IC}$). Abscissa, time; ordinate, per cent light transmission. Arrow indicates introduction of material. AS, active supernatant; IC, initial concentration. (a) Thrombocyte aggregation induced by AS ($3 \times \text{IC}$).

(b) The aggregating activity of AS ($3 \times \text{IC}$) is maintained after heating at 56°C for 30 min.

(c) The aggregating activity of AS ($3 \times \text{IC}$) is abolished after heating at 80°C for 30 min.

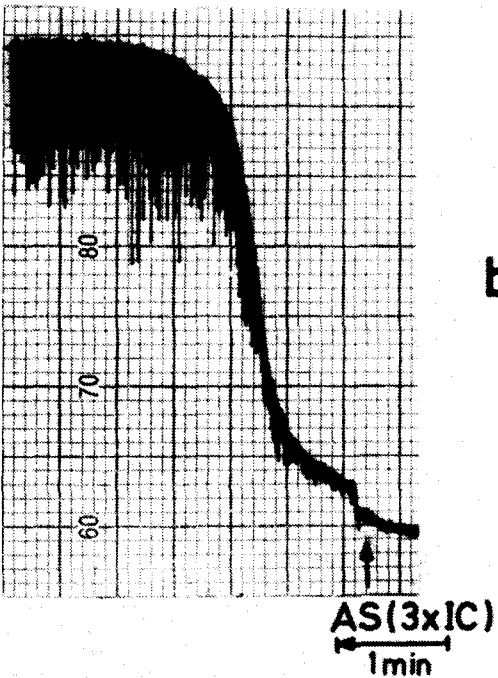
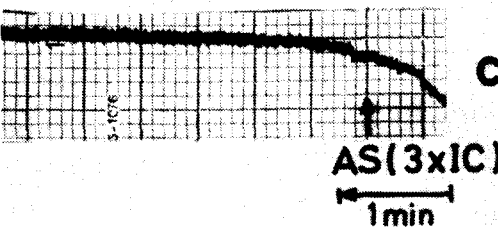
**b****c**

TABLE 2

Influence of Con-A Stimulated Lymphocyte Supernatants on Thrombocyte Rich Plasma (TRP) Clot Retraction.

Final conc.	% Clot retraction mean $\pm$ S.D.	<i>P</i>
Buffer control	67.3 $\pm$ 1.1	
CS (3 $\times$ IC)	66.7 $\pm$ 2.5	<i>P</i> < 0.05
AS (3 $\times$ IC)	84.0 $\pm$ 0.8	
CS (IC)	66.9 $\pm$ 1.7	<i>P</i> < 0.05
AS (IC)	77.7 $\pm$ 1.5	
CS (IC/3)	65.9 $\pm$ 1.4	n.s.
AS (IC/3)	71.8 $\pm$ 3.0	

CS: Control supernatant. AS: Active supernatant. *P*: Significance. n.s.: Non significant. IC: Initial concentration. S.D.: Standard deviation.

the thrombocyte suspension. The IC AS intermittently produced a reversible thrombocyte aggregation (Figs. 1 c and d), and IC/3 AS did not produce any aggregation. A 1–2 min preincubation at room temperature of thrombocytes with IC AS or IC/3 AS decreased or abolished the response of thrombocytes to ADP (Fig. 2) and to the aggregating action of thrombin (Fig. 3). The heating of supernatants at 56° C for 30 min (Fig. 4 b) did not modify their ability to induce thrombocyte aggregation, but they were inactivated at 80° C for 30 min (Fig. 4 c). The aggregation of thrombocytes induced by AS (3 $\times$ IC) begins after a variable latent period between 10 sec and 45 sec (Figs. 1 a, 4 a) and that induced by ADP begins immediately (Fig. 2 a).

The TRP clot retraction was significantly accelerated by two concentrations of AS 3 $\times$ IC and AS IC (Table 2). Parallel results were obtained with whole blood clot retraction technique (Table 3). Following the evolution of clot retraction in this system, the differences between AS and CS were found after 30 min and 60 min of incubation but not after 120 min, when all groups showed the same amount of retraction.

TABLE 3

Influence of Con-A Stimulated Lymphocyte Supernatants on Whole Blood Clot Retraction.

Final conc.	% Clot retraction					
	After 30 min mean $\pm$ S.D.	P	After 60 min mean $\pm$ S.D.	P	After 120 min mean $\pm$ S.D.	P
Buffer control	29.4 $\pm$ 2.7		47.8 $\pm$ 3.7		83.5 $\pm$ 0.7	
CS (3 $\times$ IC)	28.7 $\pm$ 1.2	< 0.05	48.4 $\pm$ 4.3	< 0.05	85.9 $\pm$ 2.3	n.s.
AS (3 $\times$ IC)	70.0 $\pm$ 1.4		83.3 $\pm$ 2.2		85.5 $\pm$ 0.7	
CS (IC)	30.1 $\pm$ 0.6	< 0.05	54.1 $\pm$ 8.9	n.s.	84.7 $\pm$ 1.2	n.s.
AS (IC)	53.2 $\pm$ 4.4		66.0 $\pm$ 3.6		85.2 $\pm$ 1.5	
CS (IC/3)	30.4 $\pm$ 2.8	n.s.	47.8 $\pm$ 4.5	< 0.05	85.0 $\pm$ 1.3	n.s.
AS (IC/3)	42.2 $\pm$ 8.7		57.0 $\pm$ 3.1		84.4 $\pm$ 1.5	

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

DISCUSSION

The multiple biologically active substances which are released from lymphocytes after stimulation with Con-A appear to comprise factors that can activate thrombocyte aggregation and increase clot retraction. An effect of contaminating Con-A can be excluded on basis of the control experiments used in present and previous experimental systems (1, 5, 6). The Con-A induced lymphocyte stimulation is generally assumed to mimic the lymphocyte stimulation induced by specific antigen, preferably the stimulation of T-lymphocytes, and similar lymphokine activities are released in both cases. The findings suggest that lymphokines have a new role in inflammatory tissue damage associated with cell-mediated immune reactions, since they indicate that lymphokines may be responsible for initiation of a thrombotic process.

The participation of thrombocytes in the development of thrombosis during immunological reactions has been suggested by various authors (5, 8, 9). This role could be due to

thrombocytic release of cationic proteins acting as permeability increasing factors, bactericidal factors or chemotactic factors (8, 13). Such similarities between thrombocyte and leucocyte activities are known from experiments with thrombocyte activators and inhibitors.

The thrombocyte factor release-phenomenon induced by ADP, immune complexes, other macromolecules, activated factors of complement and vasoactive amines is dependent on a receptor mechanism and resembles a secretory process (8, 9, 13). The fact that drugs known to inhibit the thrombocyte factor release were found to act similarly on lymphocytes (4, 5), could be due to a similarity between thrombocyte and leucocyte membrane receptors (7).

It is possible that the action of AS on thrombocytes is mediated through a membrane receptor, and the fact that low concentrations of AS decrease the response to ADP could be explained as being a result of competitive action on the same receptors. An argument in favour of this hypothesis is the fact that drugs interfering with cell metabolism of ATP and ADP, such as theophyllamine or dipyridamole, have an influence at the same time on both thrombocyte aggregation and lymphocyte production/release of lymphokine activity (4, 5, 7). However, other explanations are possible, e.g. that AS contains a cytotoxic factor which, producing damage, prevents the ADP-induced thrombocyte aggregation.

Lavelle et al. (11) pointed out the difference between the functional effect on thrombocytes of ADP and of their "platelet aggregation factor" (PAF). The addition of ADP produced an almost immediate aggregation, while the addition of cell supernatants was followed by 8–10 sec delay before aggregation occurred. A similar observation was confirmed in our experiments, and suggests a difference between the effector pathways of the two processes.

The acceleration of clot retraction in both the TRP and in the whole blood system could be due to thrombocyte activation. Some authors even consider the clot retraction method as a more sensitive assay for thrombocyte activation than the

turbidity aggregometric method (10). Indeed, in the present study, the clot retraction technique made it possible to obtain a quantitative estimation of the AS effect. The method seems more sensitive than the turbidimetric method, since IC AS was not active in the latter, but significantly increased the speed of clot retraction.

The TAA followed the LMIA when passed through Sephadex G-100 columns. It came out in fractions containing molecules larger than 10,000 daltons, remained active after 56° C heating, and was inactivated by 80° C. TAA has these properties in common with LMIA and other lymphokines, but further research is needed for its characterization as a component of the lymphokine system.

SUMMARY

Supernatants from Con-A stimulated human lymphocytes containing leucocyte migration inhibitory activity were measured for thrombocyte aggregation activity and for influence on thrombocyte rich plasma clot retraction and whole blood clot retraction. The lymphocyte released activity of the supernatants produced thrombocyte aggregation and an acceleration of clot retraction. The activity resisted heating at 56° C for 30 min and was inactivated at 80° C for 30 min. The active substance seems to have a molecular weight above 10,000 daltons. The findings suggest that thrombotic processes associated with cell-mediated (type IV) immune inflammation could be due, at least partially, to lymphokine effects on thrombocytes.

ACKNOWLEDGEMENTS

This work was supported by grants from The Danish Medical Research Council, The Danish Hospital Foundation for Medical Research, Region of Copenhagen, The Faroe Islands and Greenland. The authors are grateful to Johannes Gormsen, M.D., for valuable advice and help.

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