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EFFECT OF SOME DRUGS ON THROMBOPLASTIN (FACTOR III) ACTIVITY OF HUMAN MONOCYTES *IN VITRO*

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Abstract—Thromboplastin activity was induced in isolated human monocytes *in vitro* by lectins, immune complexes, endotoxins and the ionophore A 23187. The effect of promethazine, dexamethasone, cyclic AMP analogues, indomethacin and acetylsalicylic acid on the activity increase induced by the various stimulating agents was investigated. Dexamethasone had a significant inhibitory effect on endotoxin- and lectin-induced increase of thromboplastin activity. Cyclic AMP analogues inhibited the increase induced by immune complexes and also had a small but significant inhibitory effect on the endotoxin-induced increase. Promethazine enhanced the activity induced by endotoxin and by lectins. Indomethacin and acetylsalicylic acid had no effect at the concentrations used. Cytotoxic and generally activating effects of the compounds tested were monitored by determining the release of lactate dehydrogenase, lysozyme and β-glucuronidase.

Several lines of evidence suggest that monocyte procoagulant activity may be clinically important in certain types of disseminated intravascular coagulation [1–7], although direct proof is lacking. Monocytes isolated from peripheral blood of healthy persons have very low procoagulant activity. A substantial increase of procoagulant activity is seen after exposure of the cells to one of several stimuli, either endogenous such as activated complement (C3b) [5] or immune complexes [2,7], or exogenous such as endotoxins [4, 8, 9], lectins [10], or ionophores [11]. The monocyte procoagulant is immunologically and functionally indentical to human brain thromboplastin [4], which consists of a specific integral membrane glycoprotein, apoprotein III, of molecular weight about 52,000 [12, 13], forming a complex with phospholipids [13, 14]. The activity increase is completely inhibited by cycloheximide and to a lesser extent by actinomycin D [4, 5, 7], but it is presently unclear if actual synthesis *de novo* of apoprotein III is involved. In a series of experiments to find effective and clinically useful inhibitors of this development of thromboplastin activity, we have studied the effect *in vitro* of the prostaglandin synthesis inhibitors indomethacin and acetylsalicylic acid, the antihistaminic (H_1-receptor blocking) promethazine, the glucocorticoid dexamethasone and analogues of cyclic AMP on the procoagulant activity of human monocytes cultured in the presence of various stimulating agents. Promethazine renders partial protection against endotoxin-induced shock in mice (Ferluga, personal communication). Cortisone acetate was shown by Thomas and Good [15] to substitute for the first of the two endotoxin doses which provoke the Shwartzman phenomenon in rabbits, and Robinson *et al.* [16] recently reported that sonicated peritoneal macrophages from rabbits had increased procoagulant activity after *in vivo* administration of endotoxin and cortisone acetate. Maynard *et al.* on the other hand, reported that dexamethasone inhibited the procoagulant activity increase induced by subculturing of WISH amnion cells [17]. To monitor general activating and cytotoxic effects of the compounds used we have also determined the release of lactate dehydrogenase, lysozyme and β-glucuronidase from the monocytes.

MATERIALS AND METHODS

Promethazine was kindly given by Weider Pharmaceuticals, Oslo, indomethacin by Dumex, Copenhagen, and A 23187 by Dr. R. L. Hamill, Lilly Research Corporation, Indianapolis. Indomethacin and A 23187 were dissolved in ethanol and diluted 150–400 fold in medium before use. Concanavalin A (Con A), wheat germ agglutinin (WGA), cyclic AMP, N^6, 2-O'-dibutyryl-cyclic AMP (dbcAMP), 8-bromo-cyclic AMP (brcAMP), sodium pyruvate, β-NADH, dried *Micrococcus lysodeicticus* cells, phenolphtalein glucuronate, dexamethasone and acetylsalicylic acid (ASA) were obtained from Sigma, St. Louis, MO. Purified phytohaemagglutinin (PHA) HA 16 was obtained from Wellcome Research Laboratories, Beckenham. Endotoxin was *E. coli* 0111:B4 or *E. coli* 055:B5 obtained from Difco Laboratories, Detroit.

Human monocytes were isolated from leukocyte preparations obtained from healthy donors by the use of a cell separator (Haemonetics Blood Cell Processor Model 30) operated to separate thrombocytes and leukocytes. The isolation procedure and culture conditions have been described previously [4, 7]. The yield was regularly $0.9–3.6 \times 10^8$ mononuclear cells and $2–10 \times 10^7$ cells were plated per tissue culture dish in various experiments. About 8–15 per cent of these cells adhered to the dish under the conditions used; of the adherent cells more than 90 per cent (usually more than 95 per cent) were monocytes as evidenced by phagocytosis and nonspecific esterase staining. Differential counting of

May–Grünwald–Giemsa stained preparations confirmed that regularly more than 95 per cent of the cells had monocyte morphology. After incubation the cultures were rinsed ten times with ice cold saline and scraped off into 1 ml barbital-buffered saline, pH 7.35. The preparations were frozen and thawed and homogenized with ten strokes in a Teflon–glass homogenizer before testing for thromboplastin in triplicate in a one stage Quick system [14] and for marker enzyme activity. Thromboplastin coagulation times were converted to units/ml by using reference curves established with dilutions of standard human brain thromboplastin, arbitrarily chosen to contain 100 units/ml. Lactate dehydrogenase (LDH, EC 1.1.1.27) was assayed as described [18]. Lysozyme (EC 3.2.1.17) and β-glucuronidase (EC 3.2.1.31) were assayed according to Gordon *et al.* [19] and Gianetto and de Duve [20], respectively. Lysozyme is given as egg white lysozyme equivalents (μg/mg cell protein) and β-glucuronidase as phenolphtalein released (μg/mg cell protein).

Protein [21] and deoxyribose [22] were estimated using bovine serum albumin (Sigma) and deoxyribose (Sigma) as standards. Human serum albumin and transferrin (Sigma) and the corresponding antisera (Dakopatts, Copenhagen) were mixed to allow formation of immune complexes at antigen/antibody equivalence for 90 min at room temperature.

RESULTS

Unstimulated human monocytes *in vitro* have very low procoagulant activity [4, 5, 8, 9]. We used lectins (PHA, WGA, Con A) [10], endotoxin [4, 8], the divalent ionophore A 23187 [11], and immune complexes (transferrin–antitransferrin and albumin–antialbumin) [7] as stimulants. The antigens and antibodies were screened for absence of stimulatory activity by adding them separately to monocyte cultures. The final concentration of Ca^{2+} in the medium was 1.2 mM. There were great variations in response to the same inducer among different monocyte preparations (cf. Tables 1 and 2). Each experimental culture was therefore matched with control cultures from the same batch of cells. None of the potential inhibitors used had any significant effect on the thromboplastin activity of unstimulated monocytes in the doses applied.

Promethazine

Promethazine at a final concentration of 0.1 mM had a moderate further enhancing effect on the increase of thromboplastin activity observed in the presence of various endotoxin preparations (Table 1). At a final concentration of 0.2 mM the response to endotoxin was even more enhanced, but under these conditions a cytotoxic effect, i.e. release of LDH to the culture medium, was observed.

A similar enhancing effect of promethazine on thromboplastin activity was seen when cultures were stimulated with lectins (Table 1). The mean activity increase induced by lectins alone was about 10 fold [1052 per cent ± 87 (S.E.M.)] whereas the combination of a lectin and promethazine enhanced the response about 18 fold (1896 per cent ± 362) (Table 1). The lectins were used at a final concentration of 25 μg/ml, at which Con A and WGA give a slight inhibition of the test system [10]. The present data suggest that WGA + promethazine is the most powerful combination tested. Essentially no effect was seen at the same promethazine concentrations when immune complexes or A 23187 were used as stimulants. The mean observed activities were 91 per cent and 95 per cent of the mean activities of cultures with the respective stimulant without promethazine (Table 1).

Table 1. Effect of promethazine on increase of monocyte thromboplastin activity *in vitro*

Stimulant	Promethazine (final concentration) 0.1 mM	Number of cultures	Thromboplastin activity (% of unstimulated control ± S.E.M.)
PHA	−	6	1037 ± 110
	+	6	1534 ± 489
WGA	−	4	1290 ± 269
	+	4	2771 ± 864
Con A	−	2	836
	+	2	1186
Endotoxin	−	8	298 ± 31
	+	4	332 + 43
	+*	8	509 ± 28
Immune complexes	−	4	301 ± 37
	+	4	297 ± 11
	+*	2	231
A 23187	−	4	319 ± 61
	+	4	284 ± 57
	+*	2	343

The final concentrations were lectins 25 μg/ml, endotoxin 25–50 μg/ml, and A 23187 0.25 μg/ml. Immune complexes (transferrin–antitransferrin) corresponding to 3.7 μg antigen/ml (final concentration) were used.

* Final concentration 0.2 mM.

Table 2. Effect of dexamethasone on increase of monocyte thromboplastin activity *in vitro*

Stimulant	Dexamethasone (10 μg/ml)	Number of cultures	Thromboplastin activity (% of unstimulated control ± S.E.M.)
PHA	−	4	278 ± 38
	+	4	199 ± 4
Con A	−	4	418 ± 48
	+	4	234 ± 19
A 23187	−	4	310 ± 68
	+	4	310 ± 76
Immune	−	8	436 ± 61
complexes	+	8	419 ± 52
Endotoxin	−	10	315 ± 24
	+	10	218 ± 19

The final concentrations of stimulants were as in Table 1, except for immune complexes where the amount added corresponded to 7.5–15 μg of antigen/ml.

Dexamethasone

Dexamethasone at a final concentration of 10 μg/ml inhibited the activity induced by endotoxin (to 69 per cent ± 5) and that of the lectins used (PHA and Con A) (to 65 per cent ± 6) (Table 2). Triamcinolon had a similar effect when tested against Con A.

Dexamethasone had no effect on the increase induced by immune complexes or A 23187 (Table 2). Promethazine counteracted the inhibitory effect of dexamethasone on the activity increase induced by endotoxin (data not shown).

Cyclic AMP and analogues

Dibutyryl-cyclic AMP at a final concentration of 1 mM caused a marked reduction in thromboplastin activity of monocytes stimulated with immune complexes (Table 3). A moderate inhibitory effect on endotoxin-induced thromboplastin increase was also seen. No effect was seen when cultures were stimulated by lectins (PHA and Con A) or A 23187. A similar pattern was obtained with 8-bromo-cyclic AMP.

Cyclic AMP (final concentration 1 mM) had no significant effect on the thromboplastin activity of monocytes stimulated by immune complexes or PHA (data not shown).

Indomethacin and acetylsalicylic acid

Indomethacin at 0.1 mM was without significant effect on the increase of thromboplastin activity regardless of the stimulant used (data not shown). In a more limited series of experiments ASA (0.1 mM) was equally ineffective.

Effect of stimulants and drugs on release of cellular enzymes

Culture supernatants and cell homogenates were assayed for lysozyme, LDH and β-glucuronidase. A marked increase in release of both lysozyme and β-glucuronidase was seen in cultures stimulated with immune complexes (Table 4). A 23187 increased slightly the release of β-glucuronidase. A moderately increased release of lysozyme was seen in cultures stimulated with A 23187 and endotoxin, whereas lectins caused no release significantly above the con-

Table 3. Effect of cyclic AMP-analogues on increase of monocyte thromboplastin activity *in vitro*

Stimulant*	Additions†	Number of cultures	Thromboplastin activity (% of stimulated control‡)
Endotoxin	dbcAMP	2	76
	brcAMP	2	71
Immune complexes	dbcAMP	6	36
	brcAMP	2	44
A 23187	dbcAMP	4	100
	brcAMP	2	133
PHA	dbcAMP	4	111
	brcAMP	2	115
Con A	dbcAMP	2	95

* The final concentrations of stimulants were as in Table 1, except for immune complexes (albumin–antialbumin) where the amount added corresponded to 9–12 μg of antigen/ml.

† The final concentration of analogues was 1 mM.

‡ The stimulation with endotoxin was 1.9–3.7 -fold, immune complexes 3.3–8.0 -fold, A 23187 2.4–3.1 -fold and lectins 1.7–2.6 -fold.

Table 4. Release of lysozyme and β-glucuronidase from stimulated monocytes

Stimulant*	Lysozyme (% of unstimulated control ± S.E.M.)	Number of cultures	β-glucuronidase (% of unstimulated control ± S.E.M.)	Number of cultures
Endotoxin	171 ± 49	22	100 ± 22	20
Immune complexes	872 ± 142	20	465 ± 84	20
A 23187	365 ± 116	28	163 ± 62	22
PHA	101 ± 34	10	176 ± 62	8
Con A	93 ± 30	16	63 ± 18	16

* Final concentrations as in Table 1.

Table 5. Effect of promethazine and cyclic AMP-analogues on release of lysozyme and β-glucuronidase from stimulated monocytes

Stimulant*	Additions†	Number of cultures	Lysozyme (% of stimulated control ± S.E.M.)‡	β-glucuronidase (% of stimulated control ± S.E.M.)‡
Endotoxin	Promethazine	8	607 ± 192	505 ± 204
	dbcAMP	2	32	17
	brcAMP	2	83	82
Immune complexes	Promethazine	4	143 ± 7	127 ± 204
	dbcAMP	4	50 ± 9	42 ± 12
	brcAMP	2	93	69
A 23187	Promethazine	6	332 ± 67	475 ± 206
	dbcAMP	4	232 ± 36	277 ± 95
	brcAMP	2	111	89
Lectins	dbcAMP	6	35 ± 22	57 ± 2
	brcAMP	2	50	65

* Final concentrations as in Table 3.

† Promethazine, final concentration 0.1 mM, dbcAMP and brcAMP final concentration 1 mM.

‡ Controls stimulated with endotoxin released lysozyme 1.55 ± 0.60 μg/mg cell protein/h and β-glucuronidase 0.39 ± 0.16 μg/mg cell protein/h. The corresponding values for immune complexes were 11.05 ± 0.89 and 1.97 ± 0.38, for A 23187 3.52 ± 1.10 and 0.44 ± 0.11 and for lectins 1.08 ± 0.24 and 0.25 ± 0.13. Unstimulated controls released 0.80 ± 0.54 μg of lysozyme and 0.17 ± 0.05 μg of β-glucuronidase (for definition of units see Methods).

trol level. When immune complexes and A 23187 were used as stimulants the total content of lysozyme per culture was raised after 8–10 hr to 230 per cent ± 24 (S.E.M.) and 136 per cent ± 13 (S.E.M.) of that of incubated, unstimulated controls. In cultures stimulated with endotoxin or lectins the total content of lysozyme was not significantly different from that of the controls.

Promethazine caused additional release of lysosomal enzymes with all three types of stimulants tested (Table 5). The total lysozyme content per culture was also increased (1.8–2.4-fold) irrespective of whether the concomitant stimulant by itself caused increased lysozyme synthesis or not. The total amount of β-glucuronidase per culture was unchanged in all of these short-time experiments. Dexamethasone in most cases exerted an inhibitory effect on release of β-glucuronidase, but had essentially no effect on lysozyme release (data not shown). Prostaglandin synthesis inhibitors (indomethacin and ASA) had no significant effect on release of either enzyme.

DbcAMP inhibited lysosomal release induced by immune complexes, endotoxin and lectins (Table 5). BrcAMP was less effective in this respect. The slight to moderate increase in the release of lysozyme and β-glucuronidase caused by A 23187 alone was further enhanced 2–3 fold by dbcAMP (Table 5).

Release of the cytosol marker LDH above control levels was only seen when cells were exposed to both endotoxin and promethazine (0.2 mM final concentration), and under all other experimental conditions used more than 95 per cent of the cells excluded trypan blue.

DISCUSSION

Various compounds which interact with the plasma membrane of monocytes induce the increase of thromboplastin activity which is mainly localized in this membrane. The process is inhibited by cycloheximide and may involve de novo synthesis of apoprotein III, the protein component of tissue thromboplastin. An increased amount of apoprotein III antigen is found in homogenates of stimulated monocytes (Lyberg and Prydz, in preparation), suggesting again that apoprotein III is synthesised or made available from a precursor. The absence of any significant effect of indomethacin and ASA on this process indicates that the prostaglandin system is not involved in the response to any of the four classes of stimulants used. The doses applied were sufficient to inhibit platelet and endothelial cell cyclooxygenase [23].

In contrast, dexamethasone and promethazine showed an interesting difference with regard to action and a similarity with regard to specificity for the inducers (Tables 1 and 2). Dexamethasone inhibited the stimulation by lectins (PHA and Con A) and by endotoxin (Table 2). Promethazine enhanced markedly the effect of lectins and also the endotoxin effect (Table 1). Dexamethasone counteracted the enhancing effect of promethazine in endotoxin-induced cultures. Neither dexamethasone nor promethazine had any significant effect on the activity increase induced by immune complexes or A 23187. The effect of dexamethasone is consistent with the observations by Maynard *et al.* [17], but is apparently contrary to the effect of cortisone acetate in rabbits [16]. Steroid pretreatment for 4 days *in vivo* increased the procoagulant activity induced in peritoneal macrophages by a subsequent intravenous endotoxin injection one hour prior to macrophage isolation [16]. Difference in species, type of compound, and way of exposure to the drug may explain the varying results. The dexamethasone effect in our experiments may be mediated via an increase of cAMP [24].

The stimulatory, inhibitory or enhancing effects described here occurred without any consistent corresponding pattern of enzyme release, except that evidence for cytotoxicity (LDH release and trypan blue uptake) was only found when 0.2 mM promethazine was combined with endotoxin. The effects of immune complexes [25, 26], glucocorticoids [27, 28] and exogenous cyclic AMP [29–32] on release of lysosomal enzymes are well documented and our observations are essentially confirmatory, although cyclic AMP itself at 1 mM concentration had no effect in our system and the analogues dbcAMP and brcAMP had to be used. A 23187 has been reported to cause a more pronounced release of lysosomal enzymes from macrophages [33] than observed in the present experiments. In those experiments [33] a 40-fold higher concentration of the ionophore was used, which probably explains the differing results.

Based on the different effects of the inhibitors on the various inducing agents, multiple mechanisms for triggering the thromboplastin increase may be a possible interpretation. Other results (Lyberg and Prydz, in preparation) support the same hypothesis:

1. The response to endotoxin and lectins is apparently independent of serum, whereas the response to A 23187 and immune complexes is greatly enhanced by serum [7].
2. The kinetics of the responses differ. Lectins give a maximum thromboplastin response within 4–6 hr. Monocytes require 6–8 hr to respond maximally to endotoxin and 10–16 hr to reach maximum activity when stimulated by A 23187 and immune complexes.

The results may, however, also be explained by a sequential mechanism for the induction of thromboplastin activity. The initial membrane perturbation by the various inducing agents (lectins, endotoxins) may be followed by an internal signal (Ca^{2+}?) which triggers the protein synthesis-dependent [4, 5, 7, 10] events.

If Ca^{2+} were a common second messenger in this process, one might have expected a different kinetic result, i.e. A 23817 might be expected to induce the rise of thromboplastin activity more rapidly, since its effect would be to by-pass the normal Ca^{2+}-releasing or uptake processes triggered by the other membrane-perturbing agents. It is possible therefore that the effect of immune complexes and A 23187 is indirect, e.g. mediated via a time-consuming synthesis or release of enzyme(s) contributing to the activation of thromboplastin or induction of apoprotein III synthesis.

In any case, increased thromboplastin activity is a common end point for processes triggered by a

number of compounds interacting with the monocyte plasma membrane. These processes occur without obligatory coupling to increased release of lysosomal enzymes ("activation") or LDH (cytotoxicity).

The present observations may have a bearing on the treatment of the coagulation disorders which frequently accompany (and aggravate) disease states where endotoxin or immune complexes are involved.

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