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Generation of Procoagulant Activity by Hairy Cells in Response to Endotoxin and Phorbol Esters

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Abstract—*Several investigators have suggested that hairy cells are neoplastic B lymphocytes. These cells, however, also share some biological properties with mononuclear phagocytes. A property of these cells is the capacity to generate procoagulant activity (PCA) in response to a variety of stimuli. In this study we investigated the PCA of peripheral blood hairy cells in 19 consecutive patients with hairy cell leukaemia (HCL). Monocyte-depleted blood mononuclear cells, tested immediately after isolation, expressed little, if any, activity. However, after exposure to endotoxin, a marked increase in PCA was observed (42.1 ± 8.7 vs 1.3 ± 0.2 units/5 × 10^4 hairy cells). A significant correlation was found between the number of lymphocytes/hairy cell and the level of endotoxin-induced PCA suggesting that lymphocytes potentiate the procoagulant response of hairy cells. When stimulated with 12-0-tetradecanoyl-phorbol-13-acetate (TPA), patients' cells produced about 2–8 times more PCA than endotoxin-stimulated cells. The PCA induced by endotoxin and TPA was identified as tissue factor. These findings suggest some further relationship between hairy cells and monocytes.*

INTRODUCTION

HAIRY CELL leukaemia (HCL) is a relatively rare lymphoproliferative disorder characterized by the presence of neoplastic cells with irregular, finger-like cytoplasmic projections in the bone marrow, in the spleen and, less frequently, in lymph nodes. Several investigators have suggested that these cells are most probably neoplastic B lymphocytes [1–3]. However hairy cells have a number of properties in common with monocytes including the ability to ingest latex particles or zymosan [4,5], the presence of Fc receptors for immunoglobulins [6] and the expression of OKM-1 antigen [7,8].

A property of cells of monocyte–macrophage lineage which is not shared by polymorphonuclear leucocytes and lymphocytes is the capacity to initiate and propagate coagulation pathways. Mononuclear phagocytes can respond directly or indirectly to a wide variety of stimuli *in vitro* with the production of procoagulant substances (reviewed in refs. [9–11]). The most commonly described procoagulant activity (PCA), especially in human cells, was tissue factor, although prothrombinases and factor X activators have been reported [9–15]. In addition mononuclear phagocytes can produce and/or assemble coagulation factors on their surfaces [16–18]. In this study we examined the PCA of hairy cells in a series of 19 consecutive patients with HCL. Hairy cells were found to develop potent PCA of the tissue factor type in response to endotoxin and phorbol esters.

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MATERIALS AND METHODS

Patients

Nineteen patients with HCL, 14 males and five females, aged 35–73 (mean 52) yr, who had undergone splenectomy because of pancytopenia, were studied.

Diagnosis was made on the basis of clinical and haematological findings and was confirmed by examination of bone marrow and spleen specimens. Some clinical and laboratory data are given in Table 1. Nine patients had large proportions (> 15%) of circulating hairy cells and 10 patients had less than 10% hairy cells in peripheral blood.

Severe monocytopenia was found in all but three cases.

No severe bacterial infection was present at the time of study. Results of screening studies of the plasma coagulation system including activated partial thromboplastin time, one-stage prothrom-

Table 1. Laboratory data of the caselist

Case	Age/sex	HB (g/dl)	WBC ($\times 10^9$/l)	Monocytes ($\times 10^9$/l)	Hairy cells ($\times 10^9$/l)	Platelet count ($\times 10^9$/l)
1	60 M	8.6	72	0.01	21.6	110
2	48 M	13.3	5.7	0.01	1.7	186
3	57 M	12.8	4.5	0.01	0.22	224
4	60 M	9.8	2.6	0.02	1.35	98
5	76 M	12.7	12.3	0.05	3.24	217
6	45 M	14.4	4.8	0.01	0.28	255
7	56 M	13.2	8.2	0.04	0.65	191
8	56 M	7.6	9.6	0.01	0.48	90
9	63 M	10.9	2.0	0.02	0.10	149
10	45 M	11.4	3.0	0.01	0.14	200
11	73 F	10.1	15.0	0.05	4.5	150
12	36 F	11.5	5.5	0.01	2.5	150
13	41 F	13.5	4.0	0.01	0.3	250
14	42 M	13.3	7.3	0.02	0.2	270
15	51 M	12	3.2	0.06	0.1	170
16	48 M	11	3.3	0.01	0.5	180
17	56 F	14	16.5	0.30	11.5	230
18	50 F	12.8	3.5	0.10	0.2	190
19	66 F	11	12.9	0.10	0.8	150

bin time and fibrinogen levels, were within the normal range in all patients. A control group consisted of 14 apparently healthy donors, 10 males and four females, aged 31–67 yr (mean 49).

Isolation of mononuclear cells

Blood anticoagulated with trisodium citrate (0.015 M final concentration) was centrifuged for 15 min at 1300 rpm and platelet-rich plasma was discarded. Mononuclear cells were isolated from the remaining blood diluted with citrated phosphate-buffered saline [9 vol. PBS + 1 vol. 3.8% (wt/vol) trisodium citrate] by the Ficoll–Hypaque (Lymphoprep, Nyegaard, Oslo, Norway) gradients technique [19]. Cell suspensions were washed with citrated PBS and suspended in RPMI 1640 medium (Gibco, Glasgow, U.K.). In some experiments mononuclear cells were isolated from blood anticoagulated with EDTA (5 mM f.c.) washed in PBS containing 5 mM EDTA and suspended in RPMI medium. In all instances cell preparations contained more than 97% mononuclear cells and less than 0.5 platelets per nucleated cell. Patients' cell preparations were depleted of monocytes by adherence to plastic. After adherence, monocytes, identified by alpha-naphthyl butyrate esterase staining, were less than 1%, and hairy cells were 8–95% (mean 25) of the mononuclear cells. In controls, mononuclear cells contained 6–37% (mean 18%) monocytes. Cell viability, assessed by the trypan blue test, was always more than 96%.

All reagents utilized were free of endotoxin as determined by the limulus amoebocyte lysate assay (Microbiological Associates, Waltersville, MD). The sensitivity of the assay ranged in our hands between 0.1 and 0.2 ng/ml of LPS (*Escherichia coli* 0111:B4, Difco Lab., Detroit, MI).

Incubation of mononuclear cells and evaluation of PCA

To study the capacity of mononuclear cells to produce PCA *in vitro*, aliquots of cell preparations suspended in RPMI 1640 medium supplemented with 10% adsorbed AB serum were mixed with endotoxin (1 μg/ml of *E. coli* LPS, W., Difco) or with a similar volume of sterile isotonic saline and incubated at 37°C in plastic tubes. PCA generated in the incubation mixture was measured after 4 hr of incubation. In some experiments 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma Chem. Co., St. Louis, MO.) was used as a stimulus [20,21]. Cells suspended in RPMI supplemented with 10% adsorbed serum were incubated with TPA (100 ng/ml) for 20 hr at 37°C, then PCA was evaluated.

For all assays cells were disrupted by repeated freezing and thawing. PCA was measured by a one-stage plasma recalcification assay. Clotting time was determined in duplicate in prewarmed plastic tubes using the following test system: 0.1 ml cell material or buffer, 0.1 ml normal plasma and 0.1 ml 0.025 M calcium chloride. Duplicate times with cells differed by less than 5%. Buffer blank was more than 400 sec. To characterize PCA, human plasma from patients with congenital deficiency of factor VII, VIII, IX or X was used as a substrate. Results were expressed in arbitrary units by comparison of the clotting times of disrupted cells

with a standard curve of clotting times produced by dilutions of a human thromboplastin suspension. One thousand units of thromboplastin cause normal plasma to clot in 23 sec.

Factor VII activity of disrupted cells was assayed in a specific one-stage clotting assay using human brain thromboplastin and factor VII-deficient plasma.

Serum was adsorbed with $BaSO_4$ (100 mg/ml) for 15 min at 37°C with frequent mixing. The procedure was repeated 2–3 times. Adsorbed serum was devoid of factors VII, IX and X (< 0.001 U/ml).

RESULTS

PCA was first measured in mononuclear cell suspensions immediately after isolation (basal PCA). Patients' cells contained very low PCA (mean ± SE: 0.92 ± 0.13 units/5 × 10^4 hairy cells, $n = 19$) comparable to that of cells from control subjects (0.94 ± 0.18 units/5 × 10^4 monocytes, $n = 14$). Incubation in short-term culture without any stimulant did not significantly affect PCA (1.27 ± 0.18 vs 0.92 ± 0.13 units/5 × 10^4 hairy cells, $n = 19$, $P > 0.1$). However, after exposure to endotoxin, a marked increase in the average PCA was detected (42 ± 8.7 vs 1.27 ± 0.18 units/5 × 10^4 hairy cells, $n = 19$) (Fig. 1). For comparison, after incubation with endotoxin, mononuclear cells from 14 normal subjects responded with a PCA of 96.9 ± 23.1 units/5 × 10^4 monocytes, which was significantly higher than that of patients' cells ($P < 0.01$). Both in patients and controls the generation of PCA was almost completely abolished (> 95% inhibition, $n = 4$) by cycloeximide and actinomycin D (5 μg/ml) suggesting that protein synthesis is required for this phenomenon; these reagents did not affect cell viability at the concentrations used.

The procoagulant response to endotoxin varied widely among patients (Fig. 1). A significant correlation was found between the number of lymphocytes/hairy cell and the level of endotoxin-induced PCA in patients' cell preparations ($r = 0.54$; $P < 0.05$).

In six patients we also studied the procoagulant response of peripheral blood mononuclear cells to TPA. As shown in Table 2, TPA-stimulated cells produced about 2–8 times more PCA than endotoxin-stimulated cells.

To characterize PCA, endotoxin and TPA-stimulated cells were tested in plasmas selectively deficient in one clotting factor (Table 3). PCA was expressed to similar extents in normal factor VIII- (not shown) and factor IX-deficient plasmas. Assays with factor VII-deficient plasma produced considerably less activity. Nevertheless some PCA was consistently detectable suggesting that procoagulants other than tissue factor were generated in our experimental conditions. As it has been shown that endotoxin-stimulated monocytes may express functional factor VII/VII_a [18], we measured factor VII activity in patients' and controls' cell preparations. In the six patients studied factor VII activity was 2–12‰ (mean 8‰) of the activity of normal plasma in endotoxin-stimulated samples and 3–26‰ (mean 14.5‰) in TPA-stimulated samples. Normal mononuclear cells expressed 5–13‰ (mean 7.4‰) and 7–13‰ (mean 8.3‰) when stimulated with endotoxin

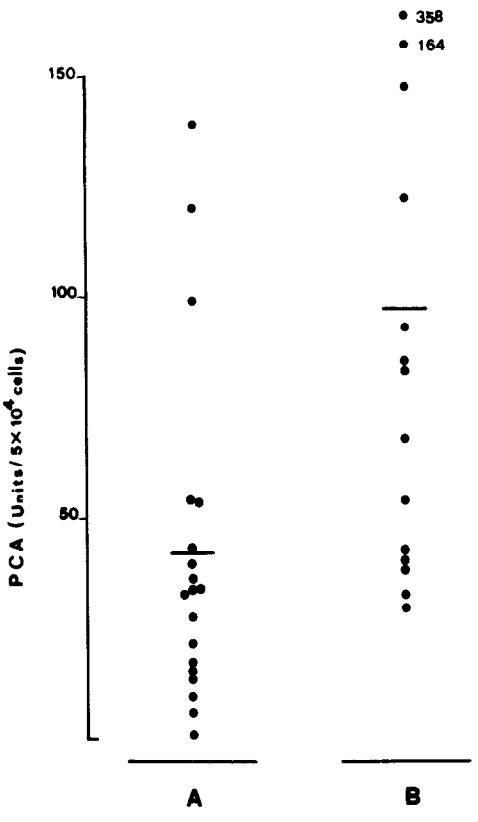

Fig. 1. PCA generated by monocyte-free blood mononuclear cells of HCL patients (A) and by mononuclear cells of control subjects (B) stimulated with endotoxin. PCA is expressed as units/5 × 10^4 hairy cells in patients and as units/5 × 10^4 monocytes in controls.

Table 2. Procoagulant response of patients' mononuclear cells to endotoxin and TPA

Patient	PCA (units/5 × 10^4 hairy cells) Endotoxin	TPA
1	37.1	71.7
2	21.3	53.1
3	28.4	95.7
4	17.3	69.3
5	5.3	39.8
6	43	149.9

Cells preparations were incubated with endotoxin or TPA at 37°C for 20 hr before PCA assay. PCA is expressed as units/5 × 10^4 hairy cells.

Table 3. Characterization of PCA *produced by endotoxin- and* TPA*-stimulated patients' cells*

Sample	PCA (units/5 × 10^4 hairy cells)		Tissue factor source (n = 3)*
	Endotoxin (n = 14)	TPA (n = 5)	
I.1Tested in normal plasma	44.1 ± 8.2	65.9 ± 9.4	70.0
II. Tested in factor IX-def. pl.	43.9 ± 7.8	64.7 ± 8.5	66.5 ± 2.3
III. Tested in factor VII-def. pl.	6.5 ± 1.7	13.9 ± 4.3	2.9 ± 1.1
IV. Tested in factor X-def. pl.	< 1	< 1	< 1
V. Treated with antiapoprotein III serum†	1.9 ± 0.3	3.2 ± 1.0	2.2 ± 0.6
VI Treated with normal goat serum†	43.4 ± 7.5	63.9 ± 8.3	69.1 ± 2.5

* Human brain thromboplastin diluted to achieve a PCA of 70.0 units.

† Samples (n = 3) were incubated with a goat antiserum to apoprotein III or normal goat serum (1:20 final dilution) for 1 hr at room temperature before testing in normal plasma; the sera were adsorbed with 10 mg/ml $BaSO_4$ for 15 min before use; after adsorption they contained less than 1% of factors of prothrombin complex.

and TPA respectively (n = 5). No factor VII activity (< 1‰) was detected in both patients' and controls' unstimulated cell preparations. Results were similar when mononuclear cells collected and washed in the presence of 5 mM EDTA were used (n = 3, data not shown).

Table 3 also shows that PCA produced by patients' cells was neutralized by a goat antiserum against the apoprotein III component of human brain thromboplastin (kind gift of Prof. H. Prydz, Research Institute for Internal Medicine, Rikshospitalet, Oslo, Norway).

DISCUSSION

A variety of malignant cells have been shown to express procoagulant activities whereby they can initiate blood coagulation. Two main types of procoagulants have been described: tissue factor and cancer procoagulant, an enzyme that directly activates factor X [22,23]. Tissue factor has been reported in human leukaemic promyelocytes and less frequently in cells from patients with other types of acute, non-lymphoid leukaemia [24–26].

An important feature of most malignant cells with respect to PCA is that they do not require any trigger but express PCA constitutionally.

This study shows that monocyte-free peripheral blood mononuclear cells from patients with HCL, containing variable amounts of hairy cells, express little, if any, PCA when assayed immediately after isolation.

It is now well established that, among peripheral blood leucocytes, the monocyte is the only cell category capable of producing PCA when triggered *in vitro* by a variety of stimuli [9–11].

Since hairy cells have been reported to share several properties with the monocytes [4–6], we investigated whether they are able to produce PCA *in vitro*. Our results show that peripheral blood mononuclear cells from patients with HCL, thoroughly depleted of monocytes, do generate PCA when incubated *in vitro* with bacterial endotoxin, a model stimulus for the production of monocyte PCA. The procoagulant response to endotoxin varied widely among different patients. In some it was vigorous, generating PCA to a level nearly comparable to the response of normal monocytes, and in a few cases it was very weak. Hairy cells were most probably responsible for PCA production since it is well established that normal lymphocytes [9–11] and some pathologic lymphocytes [27] lack this property. This conclusion is further supported by the observation that, in three patients whose mononuclear cell preparations contained more than 90% hairy cells, high levels of PCA were generated in response to endotoxin (63.1 ± 18.6 units/5 × 10^4 cells). However, the possibility that 'abnormal' lymphocytes in hairy cell leukaemia could produce PCA cannot be excluded.

The PCA generated by endotoxin-stimulated hairy cells was identified as tissue factor since it required factor VII for its full expression and was neutralized by an antiserum against the apoprotein III component of human brain thromboplastin. The minute amounts of factor VII activity associated with stimulated hairy cells most probably accounts for the PCA expressed in factor VII-deficient plasma.

Lymphocytes reportedly provide 'help' for monocyte PCA induction by various agents including endotoxin [9,10], although the requirement for lymphoid cells is not absolute [9,11,15]. In this study a significant correlation was found between the number of lymphocytes/hairy cell and the level of endotoxin-induced PCA in patients' preparations. This suggests that lymphocytes may greatly

facilitate the procoagulant response of hairy cells. It is worth mentioning in this context that, according to some investigators [28,29] immature or poorly differentiated monocytoid cells augment their procoagulant response considerably in the presence of collaborating lymphocytes, whereas mature monocytes–macrophages appear essentially independent of any helper effect of lymphocytes.

Recent studies [20,21] have shown that peripheral blood cells from patients with acute or chronic myeloid leukaemia and the human promyelocytic cell line HL 60 can be induced to differentiate into macrophage-like cells by TPA; this phenomenon is closely associated with the generation of PCA of the tissue factor type. Cells from patients with acute lymphatic leukaemia and some lymphoid cells, when treated with TPA, neither differentiate nor produce PCA. It has been suggested that the production of PCA is additional evidence of the similarity between TPA-induced macrophages and normal monocyte–macrophage cells and may represent a marker for monocyte differentiation. In the six patients studied here, the procoagulant response of hairy cells to TPA was 2–8 times higher than the response to endotoxin. Altogether our findings may indicate some further relationship between hairy cells and monocytes. Whether this means that both cells are derived from a precursor with procoagulant potential or whether the capacity to produce PCA in the hairy cells represents a function acquired by a neoplastic cell remains unclear.

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