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Aaron Polliack
Hadassah University Hospital

Dorit Gurfel
Hadassah University Hospital

Rachel Leizerowitz
Hadassah University Hospital

Helena Feinstein
Hadassah University Hospital

Yair Gazitt
Hadassah University Hospital

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PHORBOL ESTER (TPA)-INDUCED SURFACE MEMBRANE ALTERATIONS IN B-TYPE HAIRY CELL AND LYMPHOCYTIC LEUKEMIA CELLS

Aaron Polliack,* Dorit Gurfel, Rachel Leizerowitz, Helena Feinstein, and Yair Gazitt

Lymphoma-Leukemia Unit, Department of Hematology
Hadassah University Hospital and
Hebrew University-Hadassah Medical School
Jerusalem, Israel

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Abstract

This report documents phorbol ester (TPA)-induced changes in cell morphology, and in vitro growth patterns in 9 patients with hairy cell leukemia (HCL), 21 with B-type CLL and non-Hodgkin's lymphoma in leukemic phase (NHL), and 10 with acute non lymphoblastic leukemia (ANLL). TPA caused cells from HCL to adhere strongly and produce elongated cytoplasmic extensions. Many of these cells had an appearance resembling fibroblasts, while others showed marked surface ruffling and spreading containing increased dense bodies, and phagolysosomal vacuoles as seen by transmission electron microscopy.

This HCL in vitro growth pattern after TPA exposure differed from that seen in B-CLL and NHL cells, which only adhered moderately after 72 hours and readily detached in clumps. CLL and NHL-cells did not show ultrastructural features of macrophages but had either plasmacytic or HCL features. It is suggested that these different growth patterns may aid in distinguishing HCL from other B-cell neoplasias.

The expression of surface markers, tartrate resistant acid phosphatase (TRAP) and Ig secretion were studied in some B-CLL, NHL and HCL cells after exposure to different concentrations of TPA for up to 6 days. Results showed that the documented changes were frequently both dose and time dependent and the most striking HCL-features were encountered after 6 days incubation with higher concentrations of TPA. However, individual variation from case to case was noted. Nevertheless, it seems that TPA induces neoplastic B-cells to mature into secreting plasmacytoid lymphocytes, and cells with features of HCL with variable expression of surface markers and TRAP.

KEY WORDS: Phorbol esters, B-lymphocytic Leukemia, Hairy cell leukemia, B-cells, Lymphocytic leukemia, Surface membrane, glycoprotein-70.

*Address For Correspondence:
Aaron Polliack
Lymphoma-Leukemia Unit
Department of Hematology
Hadassah University Hospital
POB 12000, Jerusalem, 91120 Israel
Phone No. 427 427 (ext. 362)

Introduction

The phorbol ester TPA is an active modulator of differentiation of both lymphoid and myelogenous leukemic cells (4,5,17-20,23,24,28,31-33,35). Cells isolated from patients with acute non-lymphoblastic leukemia (ANLL) have been shown to differentiate after in vitro exposure to TPA and mature into adherent macrophages (17-20,23,31). In some cases, TPA has also been shown to induce non-B non-T acute lymphoblastic leukemia (ALL) cells along the pathway of maturation towards a more mature T or B cell phenotype (4,5,24,32,33).

In recent years TPA has also been shown to induce changes in shape, morphology and membrane antigenic components in B-type chronic lymphocytic leukemia (CLL) (3,7,8,26-28,35). Antigenic and functional alterations are reflected in the expression of surface markers which appear to be accompanied by the production of intracytoplasmic μ chain and IgM secretion (3,7,26-28,35), Furthermore, there is also an increased ability of CLL cells to stimulate in a mixed lymphocyte reaction (MLR) (26,27). Thus it seems that TPA alters the expression of a variety of cellular enzymes and surface markers in a coherent fashion and may also induce neoplastic B-cells to mature. In some of these studies (3,7,8,26-28,35) B-CLL cells developed plasmacytoid (28,35) and "hairy" cell features (3) and in some instances cells produced acid hydrolases which characterize plasma cells and other more mature B-cell neoplasias (8). Furthermore other studies have shown that cells obtained from patients with hairy cell leukemia (HCL) may also alter their shape and surface membrane after in vitro exposure to TPA, in a manner different to B-CLL (21,22,30).

The present study was performed in order to (1) further characterise the changes induced by TPA in a variety of leukemic cell types, and (2) correlate alterations in cell surface topography with changes in surface marker phenotype induced by phorbol esters. In particular, B-cells were obtained from patients with HCL, CLL and NHL in leukemic phase and compared with special emphasis on ultrastructure surface features, expression of surface membrane immunoglobulin(Ig) and the novel B-cell marker, GP-70 (10-13), the induction of tartrate resistant acid phosphatase (TRAP) and Ig secretion. Efforts were also made to define the nature of the "hairy" cell features induced in B-CLL and NHL cells after exposure to TPA and to determine whether these

changes were in any way dependent on the concentration of TPA or the length of in vitro incubation. In addition, some of the data obtained for B-CLL and NHL are contrasted with those obtained in cells isolated from patients with HCL and ANLL.

Materials and Methods

Clinical data

Nine cases of HCL, 16 with CLL, 5 with NHL in leukemic phase and 10 with ANLL were included in this study. All cases were diagnosed according to well established hematological criteria (36).

HCL: All 9 cases had the typical features outlined by Golomb (15), with 10 to 50% hairy cells in the peripheral blood. Two of the 9 cases had no palpable splenomegaly, however typical findings were found on bone marrow biopsy in all patients examined after splenectomy. Hairy cells were first identified by phase microscopy and in all cases TRAP was present in a varying proportion of cells. In all cases surface membrane immunoglobulin (SmIg) and GP-70 antigen were readily detected by immunofluorescence and relatively low numbers of MRBC-rosetting cells were seen.

CLL and NHL: Only typical cases of B-CLL and NHL in leukemic phase were included in this study (29). All cases had more than $15,000/mm^3$ leukocytes in the peripheral blood with more than 80% lymphoid cells. All cases showed positive immunofluorescence for SmIg. CLL cells usually showed faint or moderate positivity while NHL cells showed bright staining. In all cases Ia receptors and GP-70 antigen were present.

ANLL: All 10 cases had high WBC counts ($>25,000/mm^3$) with typical morphology. Cytochemical stains for myeloperoxidase, sudan black, muramidase, acid phosphatase and non-specific esterases (29) invariably showed large numbers of diffusely positive cells.

Cell Suspensions: In general, cells were isolated from the peripheral blood. In addition cells were also isolated from the spleens of 2 splenectomized HCL patients. Mononuclear cells were concentrated using the Ficoll-Hypaque gradient centrifugation technique (1), washed twice and resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum (Grand Island Biological Co., Grand Island, New York).

Electron Microscopy

TEM: Cells were fixed with 2% glutaraldehyde in phosphate buffer after initial washing with phosphate buffered saline (PBS). Cells were then postfixed in 2% osmium tetroxide, dehydrated and embedded in low viscosity epoxy resin (Polaron Ltd., England) (34) and sectioned using an MT-2 Porter Blum microtome and a diamond knife. Thin sections were mounted on copper grids, double stained with uranyl acetate and lead citrate, and viewed with a Philips EM-300 transmission electron microscope at an accelerating voltage of 40-60 kV.

SEM: Cells were initially fixed in suspension with 2% glutaraldehyde in PBS for at least one hour. Thereafter cells were prepared for SEM using the GTGO procedure as described in detail in an earlier study (9). This method involves glutaraldehyde fixation followed by tannic acid (T)-guanidine hydrochloride (G) treatment of the cells and subsequent immersion in osmium tetroxide (O) solution. The GTGO procedure results in excellent cell surface preservation and allows for easy air drying with less shrinkage than the critical point drying procedure (9). The air dried specimens were then coated by a low discharge with a thin layer of gold palladium and examined with a JEOL JSM-35 SEM at an accelerating voltage of 25-39 kV.

Incubation with TPA: 2 ml cell suspensions containing 2×10^6 cells/ml, were incubated in tissue culture dishes (35 mm diameter, Nunc, Denmark) with TPA (Sigma Chemical Co., St. Louis, Mo, USA) at a final concentration of 1, 10, 100 ng/ml. Duplicate petri dishes were incubated at 37°C in a 5% CO_2 humidified atmosphere for up to 6 days. Cells were then inspected with an inverted microscope and harvested at days 1, 2, 3, and 6. Adherence was readily visible using the inverted light microscope. Non-adherent cells readily dislodged by gentle shaking floated off the dishes into the supernatant and were detected as clumps. Both adherent and non-adherent cells were collected either directly from the medium or with a rubber policeman on days 1, 2, 3 and 6.

Surface Markers: For surface marker studies, cells were collected and washed three times with phosphate buffered saline and stained for surface membrane immunoglobulin (SmIg) with anti human Ig and for GP-70 with anti GP-70 antibody as described in earlier studies (10-13). Stained cells were scored by fluorescence microscopy and by the fluorescent activated cell sorter (FACS, 440 Becton Dickinson, USA). Culture medium was also collected and samples of 100 μl were used for determination of Ig secretion by micro Elisa test as described before (14). Briefly, aliquots of 0.1 ml of culture supernatant were placed in triplicate wells of an anti human Ig coated microtiter plate (Nunc, Denmark micro Elisa plate). Anti human Ig conjugated to alkaline phosphatase was used for detection of bound Ig (Sigma, St. Louis, MO, USA). Purified IgG (Miles Laboratories Inc., USA) was used as a standard for quantitation.

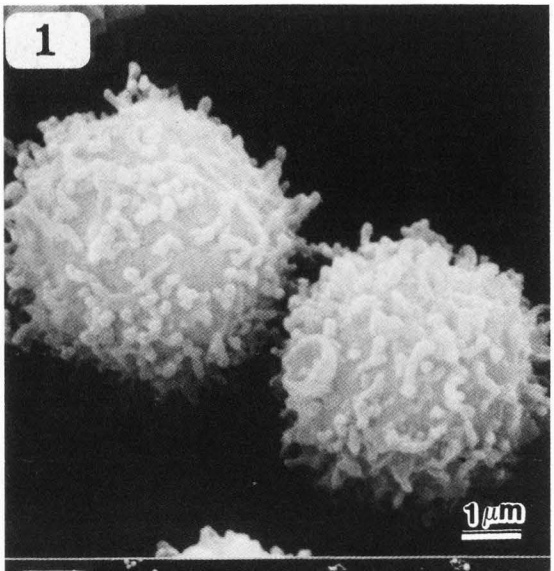
Cytochemistry: Staining for myeloperoxidase, sudan black, esterases, acid phosphatase and muramidase in leukemic cells was performed in ANLL cells prior to incubation as described in earlier studies (28,29) while TRAP staining was done according to Yam et al. (37).

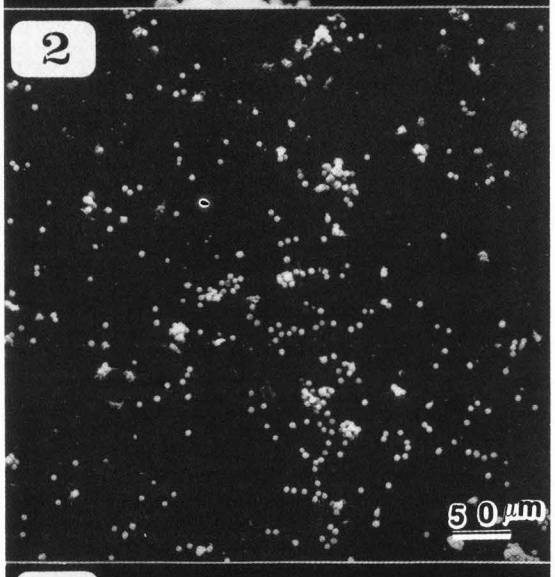
Results

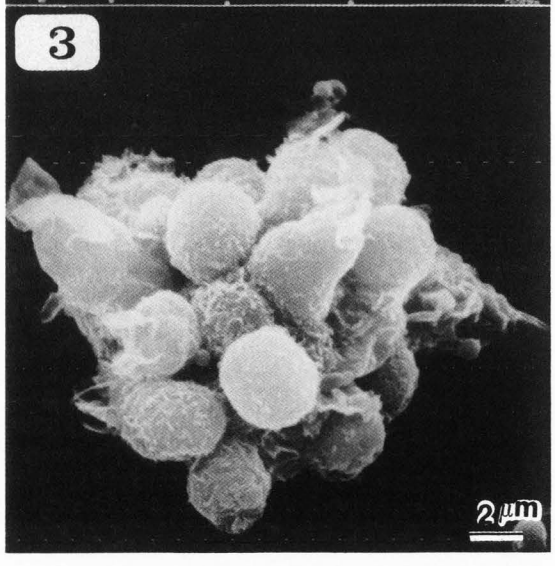
Scanning electron microscopy

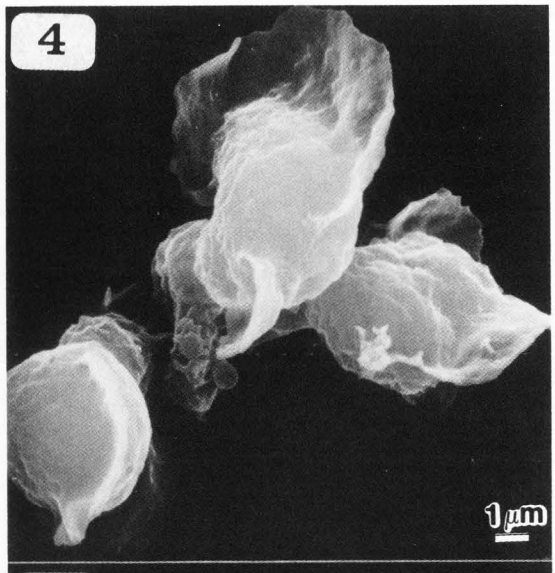
CLL and NHL: Cells from all cases examined prior to TPA exposure were spherical with varying numbers of short finger-like microvilli (Figure 1). B-NHL cells generally showed more microvilli than B-CLL cells. Control lymphocytes after incubation for 72 h were rarely adherent, generally lacked microvilli and many underwent necrosis.

After TPA exposure for up to 72 h B-CLL and NHL cells from all cases were minimally adherent, forming only small clumps or aggregates (Figure 2) which were readily dislodged from the substrate (Figure 3). After 72 h incubation with TPA, cells tended to lose their characteristic microvilli. Furthermore cells appeared less spherical in shape, with some ruffles at the periphery (Figure 4). Transmission electron microscopy showed that many









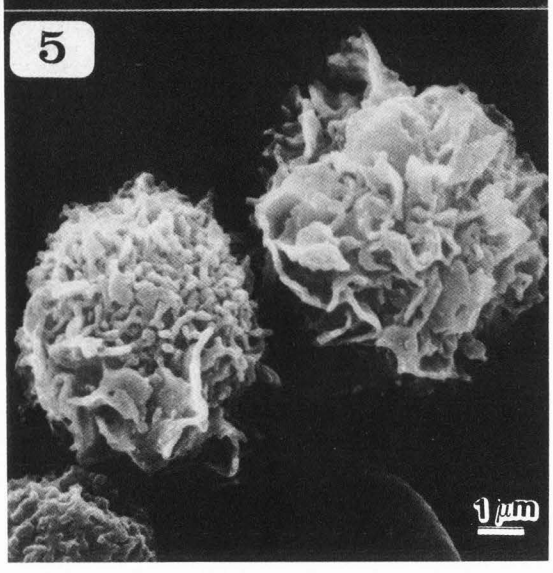


Figure 1. Spherical B-CLL cells with multiple surface microvilli.

Figure 2. Small numbers of B-CLL cells attached to the substrate after 72 h exposure to TPA.

Figure 3. Clumps of TPA-exposed B-CLL cells collected from the supernatant medium showing scattered microvilli and polarized ruffles.

Figure 4. Higher magnification of a clump of B-CLL cells collected from the supernatant medium after incubation with TPA for 72 h. Polarized peripheral ruffles are readily evident.

Figure 5. Close up of two hairy cells prior to incubation with TPA, showing typical surface features.

Table 1

ALTERATIONS IN CELL SHAPE AND MORPHOLOGY AFTER 72 H EXPOSURE TO TPA

Type of Leukemia	No. of Cases	Adherent Colonies	Non-Adherent Clumps	Elongated "Fibroblast-like"	TEM and SEM Findings
CLL & NHL	21	1	21	1	Plasmacytic and hairy cell features
HCL	9	9	0	9	No plasmacytic features. Adherent cells with ruffles
ANLL	10	10	0	1 Occasional cells	Spreading adherent macrophages with ruffles (28).

of these cells had more irregular surfaces and had developed an increase in the amount of rough endoplasmic reticulum as seen in secreting B-cells and plasma cells and as described in earlier studies (28,35). No ribosomal-lamellar complexes were seen.

HCL: Prior to incubation with TPA, HCL cells displayed the typical surface features described in earlier SEM studies (16). Cells showed the presence of prominent ruffles with clusters of microvilli scattered in between the ruffles (Figure 5). Other HCL cells showed multiple microvilli with smaller numbers of ruffles. After incubation with TPA for 72 h, HCL cells became markedly adherent and did not detach after vigorous shaking (Figures 6, 7). In all cases HCL cells underwent gross shape changes and long thin cytoplasmic processes and filopodia, which were frequently branched, became evident. These cytoplasmic processes reached up to 10 times the cell diameter (Figure 8). Many of the cells showed extensive surface ruffled folds (Figure 9). Occasionally TPA-treated cells retained their spherical shape and surface features after adherence, however control cells were rarely seen on the underlying substrate after 72 h but when present they appeared to retain their spherical shape and typical surface features. The thin elongated filopodia and spreading "macrophage" appearance were not seen.

Transmission electron microscopy showed that TPA exposed HCL cells showed more features of the macrophage cell series than TPA-exposed B-CLL and NHL cells. The plasmacytic features so typical for the latter were not evident in TPA treated hairy cells. TPA-exposed HCL cells had markedly irregular surfaces and elongated cytoplasmic extensions. Sometimes containing ribosomal-lamellar complexes, were seen (Figure 10). These HCL cells had much more abundant cytoplasm which contained more dense bodies, vesicles and phagolysosomes than HCL cells unexposed to TPA (Figure 11).

ANLL: In all cases the dominant cell type in the peripheral blood, prior to incubation with TPA, showed ridge-like folds and ruffles covering the entire cell surface (Figure 12) as described in detail in earlier studies (28). Control cells cultured without TPA rarely formed adherent colonies and only isolated cells spread out onto the substrate and became adherent. In all TPA exposed cultures there was striking adherence of the cells to the underlying substrate and vigorous shaking did not result in cell detachment. Confluent overlapping colonies of cells were sometimes seen as described for myelomonocytic leukemias in an earlier study (28). Cells spread out onto the substrate and became flattened with marked ruffling of their surfaces (Figures 13, 14). Despite the fact that some of these adherent cells resembled TPA-treated HCL colonies in culture, "fibroblast"-like cells with elongated thin cytoplasmic extensions were noted in only one case (Figure 14). In this respect they were very different from HCL cells. TEM confirmed these observations and showed that the majority of adherent TPA-treated monoblasts were in fact macrophages containing many dense bodies, vacuoles and phagolysosomes as described in an earlier study (28).

In summary (Table 1) 72 h TPA-treated HCL cultures were distinctly different from those observed in B-CLL and NHL cells, despite the fact that HCL cells are also B-derived neoplasias. The elongated "fibroblast"-like appearance of the cultures TPA-exposed HCL cells was not seen in B-CLL and NHL and was only rarely evident in ANLL cultures exposed to TPA.

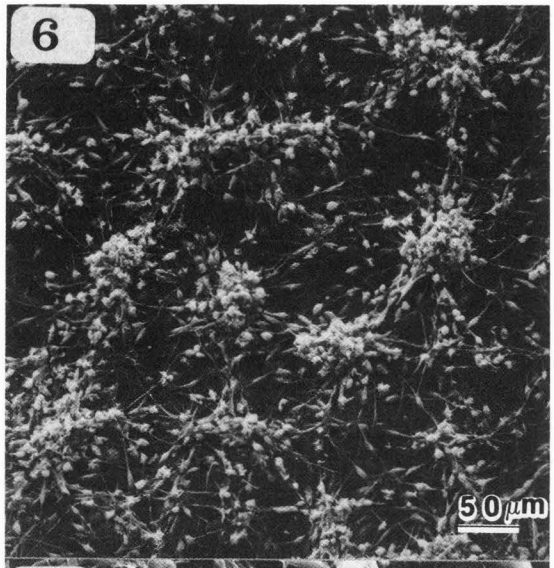
6 day B-CLL and NHL cultures: B-NHL and CLL cultures examined after 6 days exposure to TPA, showed a different growth pattern than that

Figures 6, 7. Low magnification of adherent hairy cells after 72 h incubation with TPA. Cell cultures are overlapping. Many thin elongated cytoplasmic extensions are seen.

Figure 8. Typical example of single hairy cells with fibroblast-like appearance after TPA exposure. The cells show thin elongated cytoplasmic extensions, some of which are branching.

Figure 9. TPA-exposed hairy cells showing prominent surface ruffles.

Figures 10, 11. TEM of TPA-exposed hairy cells: Close up of cytoplasm of TPA-exposed hairy cell showing ribosomal-lamellar complex (Figure 10); Figure 11 shows a TPA-treated hairy cell with abundant cytoplasm, showing multiple vesicles.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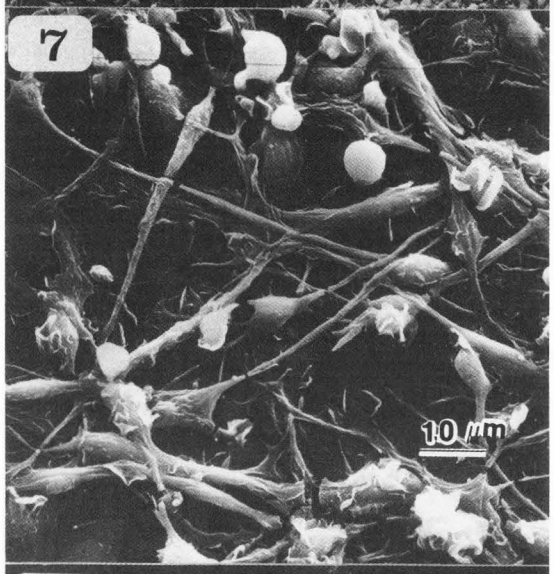
50 μm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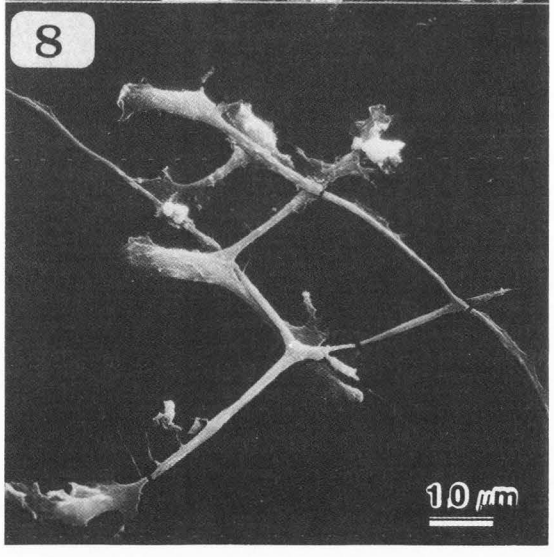
10 μm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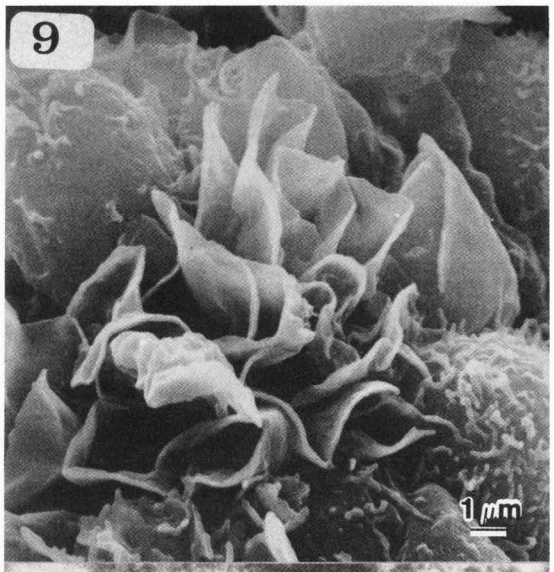
10 μm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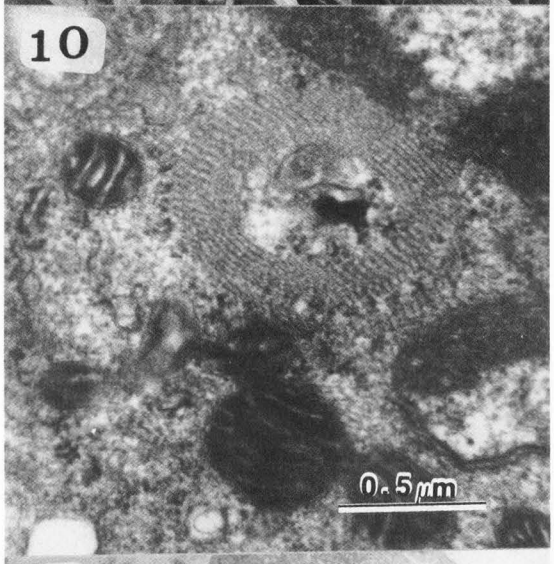
1 μm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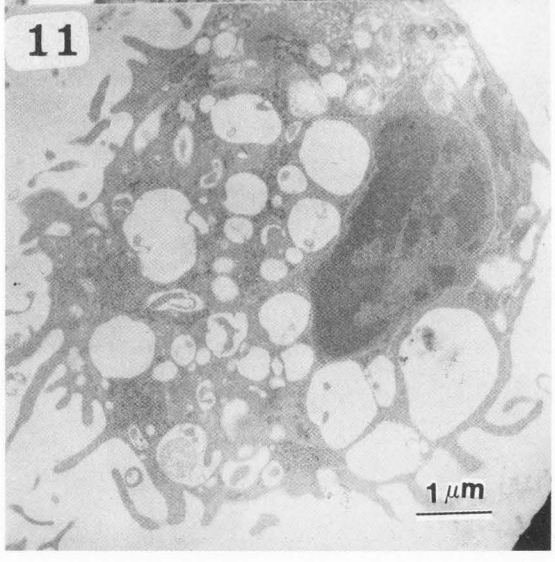
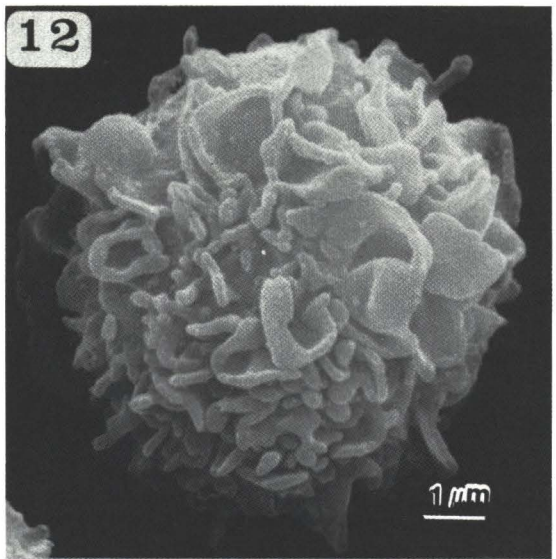
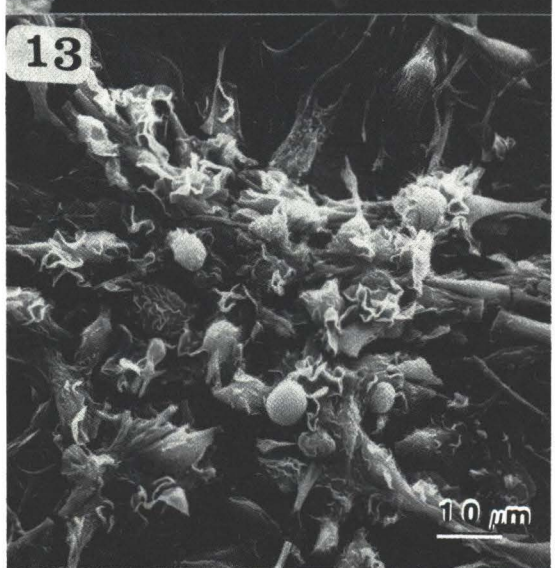
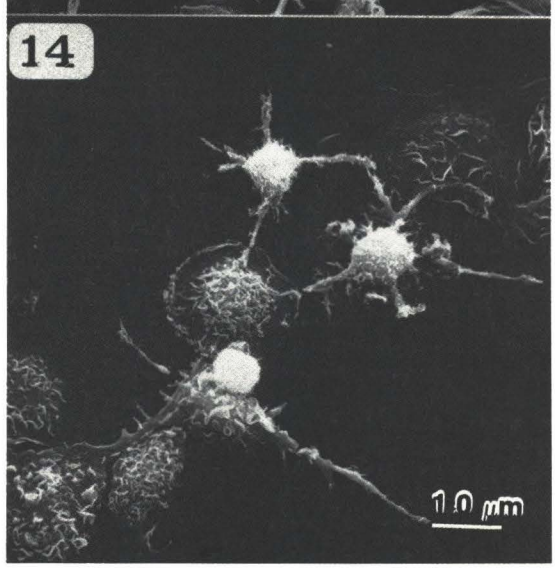
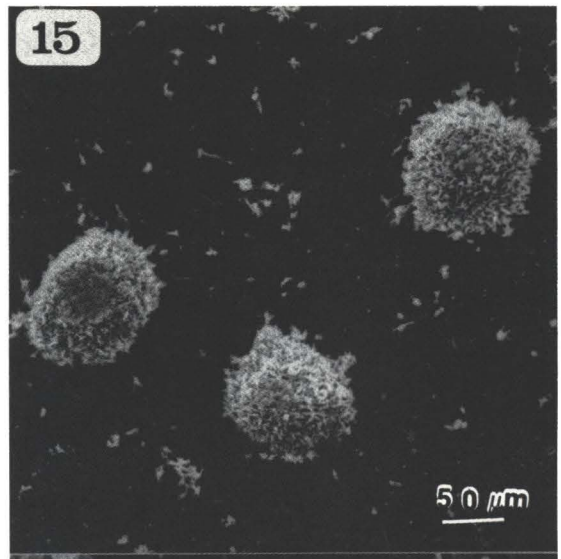
0.5 μm


11
1 μm









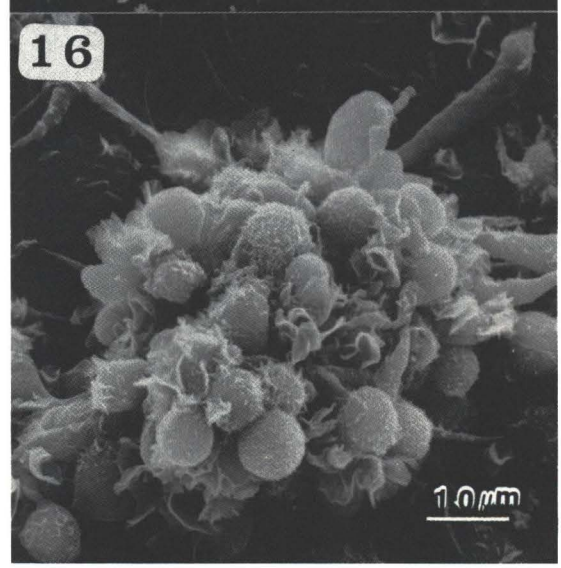
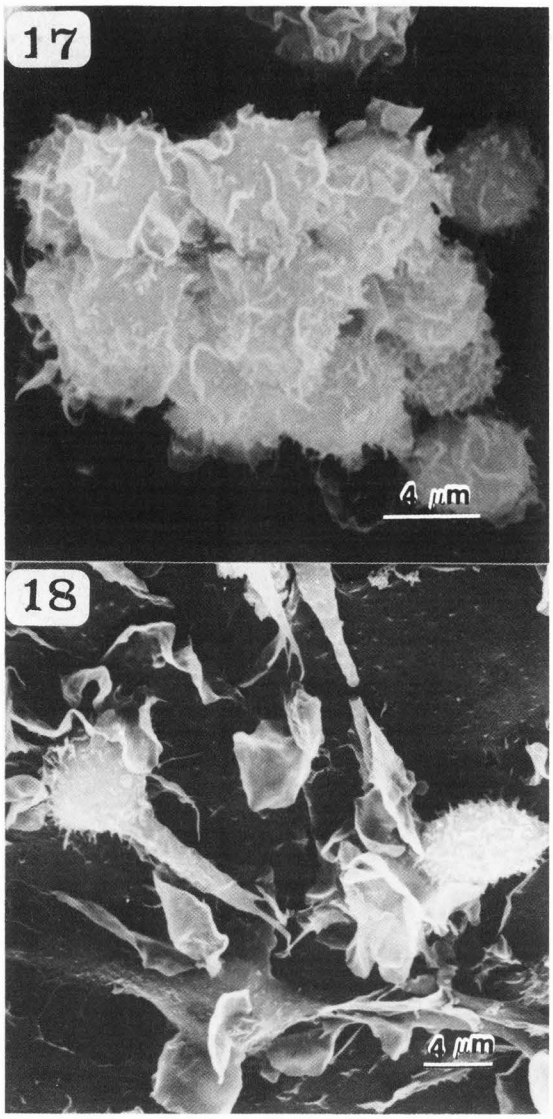


Figure 12. Leukemic monoblasts prior to incubation with TPA showing prominent transverse folds and ruffles.

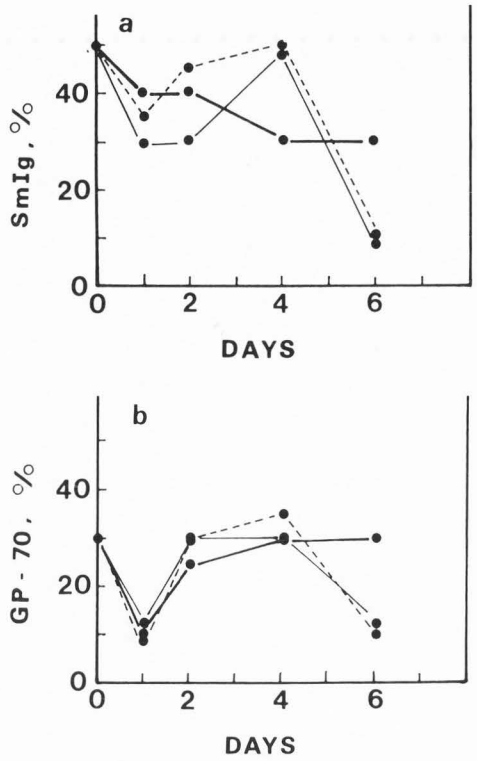
Figures 13, 14. TPA-exposed monoblast cultures, Figure 13 shows adherent spreading monocytic cell cultures with prominent surface ruffles (x 3200). Figure 14 illustrates an adherent culture with some of the cells showing elongated cytoplasmic extensions resembling those seen in HCL.

Figure 15. Low magnification showing adherence and larger clumps of B-CLL cells after 6 days culture with TPA.

Figure 16. Higher magnification of B-CLL cells spreading onto substrate with the development of prominent ruffles and hairy cell-like appearance after 6 days incubation with TPA.



Figures 17, 18. 6 day TPA-cultures B-CLL cells showing shape/change, hairy cell-like appearance and more prominent adherence onto substrate than that seen after 72 h.



Figures 19a & b. Distribution of SmIg (a) and GP-70 (b) in CLL cells (patient No. 9 in Table 2), following incubation with TPA. Cells were cultured and stained for surface markers as described in Materials and Methods section. Cells were cultured with 1 ng/cc of TPA (-); 10 ng/cc (---) and 100 ng/cc (——) of TPA. Cell fluorescence analysis and % positive cells were obtained by counting 30,000 cells in FACS (Becton Dickinson, 440) at 600 V and x 4 fluorescence amplification.

observed after 48-72 h. Cells adhered more markedly to the substrate and larger clumps were seen (Figure 15) while individual cells within these clumps spread impressively and developed prominent ruffles (Figure 16). Cells frequently developed both ruffles and small clusters of microvilli, resembling HCL cells (Figure 17), while others became elongated and irregular with cytoplasmic extensions (Figure 18). These late "hairy" cell features were more evident after incubation with the higher concentrations of TPA (10 ng and 100 ng TPA/cc) and less prominent after 1 ng/cc of TPA. They were most impressive after 6 days culture and rarely seen until day 6. Heterogeneity of cell response was noted from patient to patient and in individual cases no differences between 1 ng, 10 ng and 100 ng TPA/ml was noted while in others, marked morphological changes were evident after both concentrations were used, even after short incubation periods of 24-48 h. Plasmacytoid transformation of cells was more striking after cells were incubated for 48-72 h with TPA while "hairy" cell features were more prominent after 6 days incubation with TPA. Thus it seems that the in vitro morphological changes seen in this study were both time and dose dependent, a phenomenon which is also discussed in relation to changes in TRAP staining and surface markers.

Surface markers, Ig secretion and cytochemical changes in TRAP

Eleven CLL, 7 NHL, 5 HCL patients and 4 healthy individuals were included in this part of the study (Table 2). Mononuclear cells concentrated after gradient centrifugation were incubated with low dose (1 ng/ml), intermediate dose (10 ng/ml) and high dose (100 ng/ml) of TPA for up to 6 days. Cells were examined on days 1, 2, 3 and 6 for Ig secretion, expression of SmIg and GP-70 and for TRAP positivity.

CLL: In general, the extent of alterations recorded after TPA depended on the dose and duration of exposure to TPA. Thus, as illustrated in Figure 19, 24 h exposure to TPA induced a slight initial decrease in the expression of both surface

Table 2

TPA-INDUCED ALTERATIONS IN THE EXPRESSION OF SURFACE MEMBRANE MARKERS, TRAP AND Ig SECRETION IN CHRONIC LYMPHOCYTIC LEUKEMIA (AFTER 6 DAYS CULTURE USING 100 ng/cc TPA)

Patient number	Surface Markers SmIg (%) -TPA	SmIg (%) +TPA	GP-70(%) -TPA	GP-70(%) +TPA	TRAP % -TPA	TRAP % +TPA	Ig Secretion*
1	60	30	30	20	4	90	-
2	5	25	0	20	0	67	-
3	40	60	22	30	0	60	-
4	35	5	20	5	0	ND	-
5	55	20	30	25	0	ND	+
6	5	60	0	60	0	65	++
7	25	45	35	15	2	80	++
8	25	10	30	10	0	20	+
9	60	10	30	10	0	20	+++
10	20	0	25	0	0	ND	+
11	20	0	10	0	0	69	++
Mean ±SD	32(±20)		21(±10)		0.6(±1.3)	59±25	

*less than 50 ng/ml (-); greater than 50 -100 ng/ml (+); greater than 100 - less than 500 ng/ml (++); greater than 500 ng/ml (+++).

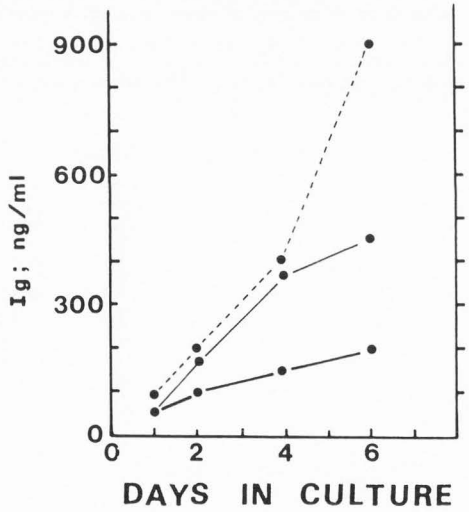


Figure 20. TPA-induced Ig secretion in CLL cells. Cell culture (patient No. 9 in Table 2) and measurement of Ig secretion were performed as described in the Materials and Methods section. Cells were cultured with 1 ng/cc of TPA (-), 10 ng/cc (---) and 100 ng/cc (——) of TPA.

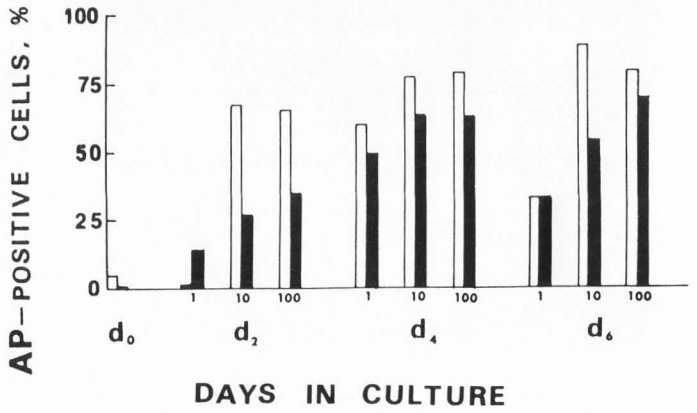


Figure 21. TPA-induced acid phosphatase in CLL cells. Cell culture (patient number 9 in Table 2). ▯ acid phosphatase, ■ tartrate resistant acid phosphatase (TRAP). Cells were cultured with 1 ng, 10 ng and 100 ng/cc of TPA for 6 days (d_0 = day zero; d_2 = day 2; d_4 = day 4; d_6 = day 6).

markers followed by a transient increase at day 4 and subsequent decrease at day 6 when the level of expression of GP-70 and SmIg was at its lowest. On the other hand Ig secretion (Figure 20) and the proportion of TRAP positive cells (Figure 21) showed a more convincing time and dose dependency for TPA. However, in general as with the surface ultrastructural changes, heterogeneity in response to TPA was also noted in relation to Ig secretion and surface marker expression as illustrated in Table 2. From this table it is evident that both SmIg and GP-70 expression before TPA treatment was highly heterogeneous which could explain why in some cases CLL cells reacted to TPA with increased expression of surface markers while others responded in the reverse fashion. Ig secretion was also heterogeneous and in less than half of the patients, the cells secreted Ig following TPA treatment while in the rest no increase was detected. In contrast to these

Table 3

TPA-INDUCED ALTERATIONS IN THE EXPRESSION OF SURFACE MEMBRANE MARKERS, TRAP AND Ig SECRETION IN NHL, HCL AND CONTROL LYMPHOCYTES (AFTER 6 DAYS CULTURE USING 10 ng/cc TPA)

Type of Leukemia	Pt. No.	Sm Ig (%) -TPA	Sm Ig (%) +TPA	GP-70 (%) -TPA	GP-70 (%) +TPA	TRAP (%) -TPA	TRAP (%) +TPA	Ig Secretion*
NHL (in remission)	1	0	10	0	5	0	5	-
	2	6	60	0	10	0	8	-
	3	12	65	0	0	0	30	-
Mean ±SD		9(±6)					14(±13)	
NHL (in partial remission or active disease)	4	0	60	0	30	0	48	+
	5	15	60	4	50	0	65	+
	6	35	13	20	12	0	52	+
	7	40	60	20	30	0	25	+++
Mean ±SD		22.5(+18)		11(±10)			47(±16)	
HCL	1	5	12	6	11	5	39	-
	2	10	65	6	0	2	10	++
	3	5	60	6	65	6	12	+++
	4	5	35	0	25	10	12	+++
	5	0	10	0	5	ND	ND	+++
Mean ±SD		5(+3.5)		3.6(+3)		5.8(+3)	18.3(+4)	
Normal	1	18	0	4	0	0	20	+
	2	20	0	3	0	0	18	+
	3	19	26	11	19	0	10	+
	4	25	12	5	6	0	10	+
Mean ±SD		20(±3)		6(±3)			15(±5)	

*less than 50 ng/ml (-); greater than 50-100 ng/ml (+); less than 500 ng/ml (++); greater than 500 ng/ml (+++). ND = not detected

findings, an impressive increase in TRAP positive cells was observed in all cases tested, although in almost all instances, varying numbers of TRAP negative cells were also found (Table 2).

NHL: Cells from seven NHL patients were examined. These patients were in complete remission (CR), partial remission (PR) or with active leukemic phase in the peripheral blood. Prior to TPA exposure the expression of surface membrane Ig, GP-70, TRAP and Ig secretion were very low in cells obtained from patients with NHL in CR. Cells derived from patients with NHL in PR and with active diseases showed more expression of surface Ig and GP-70 but were essentially TRAP negative. Following TPA exposure a moderate increase in Ig secretion, expression of surface Ig and GP-70 and TRAP positivity was noted in cells from patients with CR while in cases of PR or active disease a greater increase in the expression of surface Ig and particularly GP-70 was noted while TRAP positivity and Ig secretion were also increased, (see Table 3).

HCL: In the five instances of HCL with relatively low numbers of hairy cells in the peripheral blood the expression of surface membrane Ig, GP-70, TRAP positivity and Ig secretion was relatively low but an increase in all these parameters occurred following exposure to TPA.

Normal lymphocytes: Prior to TPA treatment normal peripheral blood lymphocytes had relatively low expression of surface membrane Ig and GP-70 while TRAP positivity was close to zero. After TPA exposure, only the level of TRAP positivity increased to a mean of about 15% with a mild increase in Ig secretion. GP-70 and surface Ig expression were not increased in the normal cells.

Discussion

Impressive and consistent changes in morphology and cell shape were observed in HCL after cells were exposed to the phorbol ester TPA. After TPA exposure hairy cells became very adherent to the underlying substrate, and were not dislodged by vigorous shaking. Cells produced extremely long narrow cytoplasmic extensions which were evident in

all cases. These adherent cells developed surface ruffles, resembled macrophages and fibroblasts, and spread onto the substrate forming confluent cultures. Transmission electron microscopic features were reminiscent of the macrophage cell series, however, cells retained their ribosomal lamellar complexes which are regarded as typical for HCL (6), despite their elongated fibroblast-like appearance. Similar findings were reported by Lockney et al. (21) who also described a unique cell surface glycoprotein expression in HCL. Despite the fact that hairy cells are known to be B-lymphocyte derived they did not develop plasmacytoid features after TPA exposure, as in other B-cell disorders. On the contrary, TPA appeared to enhance the adherent and phagocytic properties evident in HCL cells under normal conditions (6), while other B-cell characteristics such as Ig secretion were not affected. Despite the fact that CLL and NHL cells are also B-derived their in vitro growth response to TPA was very different. B-CLL and NHL cells showed minimal adherence onto the substrate after TPA exposure but did not form spreading monolayers. Cells appeared to form small clumps and were readily detached into the supernatant. Furthermore, they did not develop ultrastructural features of macrophages or fibroblasts but many acquired hairy cell and plasmacytoid features.

It is of interest to note that in general HCL cells showed a different pattern of cell growth than myelo-monocytic leukemic cells exposed to TPA. The elongated cytoplasmic filopodia and fibroblast appearance encountered in HCL were seldom seem in leukemic monomyeloblasts despite the fact that both cell types showed extensive ruffling of their surfaces and marked adhesion to the substrate, properties which are both regarded as characteristic for macrophages. The dramatic morphological changes induced in HCL cells by TPA appear to be specific for this leukemic cell type and some authors (21,22) have suggested that they are due to changes in the non-glycosylated cytoskeletal components. In the light of the in vitro growth patterns observed for HCL, which are distinctly different to those encountered in B-CLL and NHL, we continue to recommend that short term culture and exposure to TPA be added to the diagnostic laboratory tests currently employed in the differential diagnosis of HCL and other B-cell neoplasias. This laboratory test may be particularly useful to distinguish HCL from other B-type lymphoid neoplasias which may at times show clinical and cytological features mimicking HCL (2,25).

Other interesting aspects of this study relate to the apparent time and dose dependency in response to TPA in cases of B-CLL and NHL. The maximum expression of surface membrane marker alterations appears to occur after 6 days incubation and after higher concentrations of TPA. Thus TPA induces non-secreting B-cells to evolve into transformed lymphoblasts, secreting plasmacytoid cells, and plasma cells with eventual evolution into cells with features seen in hairy cell leukemia. The latter include the appearance of TRAP positivity in a high proportion of cells and surface features similar to those encountered in HCL. The above features were most prominent after 6 days incubation and 100 ng/cc TPA and less obvious after 72 h incubation and lower concentrations of TPA. Another interesting aspect was the variability expressed in almost all tested parameters in the different cases. This heterogeneity in response to TPA was also evident in the expression of SmIg and GP-70, cytoplasmic Ig secretion, and TRAP positivity as well as in surface morphology, as seen by SEM. This heterogeneity was already noted in the B-CLL and NHL cells prior to incubation with TPA and was more apparent after TPA exposure. Furthermore there appeared to be little correlation in the expression of the different cytological and immunological features regarded as characteristic for HCL, e.g., typical adherent hairy cells with characteristic surface features after TPA were TRAP negative with minimal expression of SmIg and GP-70, while in others the reverse was found. Thus it seems that differentiating agents like TPA are able to induce B-CLL and NHL cells to mature along the pathway of secreting B cells towards hairy cells. This pathway includes the development of secreting plasmacytoid lymphocytes, plasma cells and eventually cells with some of the features seem in HCL.

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Discussion with Reviewers

D. Dantchev: Did you study the morphological and other surface changes of T-cells exposed to TPA in the same experimental conditions and particularly of OKT4+ and OKT8+ cells?

Authors: In this study we have no data concerning OKT4+ and OKT8+ cells derived from the patient group described. However, recently we have started to follow T cell surface markers expression in TPA-treated B-CLL mononuclear cell suspensions. As yet we have noted no substantial differences in the expression of these markers in the cases examined.

We have no information about the morphological changes in these cells, however in all control cultures exposed to TPA in vitro, the dominant cell types were T-lymphocytes and in these cases no outstanding findings were noted.

D. Dantchev: After incubation of HCL cells with TPA for 72 h you observed in all 9 cases studied elongated cells with features of cultured macrophages and fibroblasts (Fig. 8), and also other cells producing extensive surface ruffled folds (Fig. 9): Is it the same population of pathological hairy cells or two different populations? Your cases of HCL present only 10 to 50% hairy cells: What is the nature of the other mononuclear cells?
Authors: This is indeed an important question to answer because of the difficulty in obtaining cases of hairy cell leukemia with a pure population of hairy cells in the peripheral blood. Thus one has to work with a mixed hairy cell and undefined lymphocytic population in these cases. However when we subsequently used hairy cell leukemia cell preparations concentrated from the spleens of splenectomized patients containing more than 80% hairy cells or cultured hairy cell lines, the same pattern of changes emerged which provides indirect evidence that the SEM changes described in this study are indeed those occurring in hairy cells and not in other mononuclear cells.

D. Dantchev: There is a high heterogeneity in response to TPA, expressed in the different parameters tested in 11 cases of CLL (Table 2): Is it the same in HCL, NHL, and ANLL?
Authors: As opposed to the heterogeneity seen in B-type CLL cells exposed to TPA, cells derived from patients with HCL, NHL in leukemic phase and ANLL showed consistent morphological and surface marker changes without much heterogeneity. Markers on ANLL cells were not assessed using monoclonal antibodies.

D. Soligo: Do the authors have any experience with short term cultures and TPA exposure in undifferentiated leukemias or in poorly differentiated ANLL? Is there a diagnostic role for your methodology?
Authors: As the reviewer knows from his own clinical experience, cases of undifferentiated leukemia are extremely rarely obtained in clinical practice. Over the past year we received 3 of these cases, one of which was a patient with acute leukemia showing some lineage infidelity in a small proportion of cells while most of the cells were undifferentiated. In two of the cases TPA-induced the appearance of myeloperoxidase positive blasts as seen by transmission electron microscopy. In the third case, a proportion of cells developed paranuclear acid phosphatase and early T-cell markers after TPA. Thus this methodology may have some diagnostic potential in acute leukemias of this nature.

D. Soligo: Human recombinant alpha-2C interferon has been recently shown to have a clear antitumor effect on hairy cell leukemia which still has to be elucidated. In short term cultures alpha-2C interferon was shown to induce antigen modulation in hairy cells. Induction of class II HLA antigens was also described on B-cell lymphomas and macrophage cell lines. Do you think that cell surface morphological changes could be also detected and help in some way to understand the mechanism of action of interferons on B-cell malignancies ?
Authors: We have no experience with alpha-2C interferon interactions with hairy cell leukemia or other B-cell neoplasias. However other authors have published striking morphological changes in both these disorders. One of these studies was reported in the recent SEM meeting by Gamliel et al. (39). It is indeed possible that analysis of these changes may help in understanding the mechanism of action of interferons in B-cell diseases.

M.C. Rosner: Do the authors not feel that the transformation of neoplastic B-cells into hairy cells requires more proof than that presented in the manuscript?
Authors: In this study we have been careful to use the terminology "hairy cell features" after exposure of B-CLL cells to TPA. In fact other authors have been more liberal in this respect and termed the changes seen as transformation to hairy cells. However, the surface changes recorded resemble those seen in hairy cells and the induction of $TRAP^+$ cells is most impressive. The cells transformed do not show ribosomal lamellar complexes but have other ultrastructural findings encountered in hairy cells by transmission electron microscopy. Although they lack strongly staining surface immunoglobulin they do express other hairy cell markers detected by monoclonal anti Leu M-5 and TAC, regarded as typical for hairy cells. Indeed, these cells show many features of hairy cells!

M.C. Rosner: No biochemical and surface marker data are available for the NHL, ANLL and HCL patients. Without this data are many of the claims made in the paper not justified?
Authors: The general answer is no, as we have attempted in this report to stress the value of the in-vitro growth patterns in distinguishing different leukemias. The primary question was in fact - can we distinguish HCL, from other B-cell neoplasias and ANLL? The answer to this specific question is yes, we can when samples are examined by the SEM! Some of the surface marker data obtained in NHL and HCL patients has now been included in the text of the manuscript. Data on ANLL cells from a larger series of patients has been reported in an earlier publication (text ref. 28).

J.S. Greenberger: Are the changes seen in the clumps of TPA-exposed B-CLL cells (Fig. 3) restricted to TPA or is this seen with exposure to alkylating agents (L-PAM) or other non-specific toxic agents? Do the cells also clump in a similar fashion if treated with Con A, PHA or other mitogens?
Authors: We have not used L-PAM, alkylating agents or other non-specific toxic agents in our studies until now but we do not feel that the changes illustrated relate to toxicity of the TPA. Cells appeared to be viable when the trypan-blue exclusion tests were performed and light and transmission electron microscopy confirmed that the cells were viable. Toxicity with cellular damage causes a different surface morphology which is well recognized by SEM. "Clumping" is not a feature of PHA or PWM-transformed mononuclear cells, which

in general result in a different cell surface pattern of changes. Con-A may produce similar clumping without the striking changes in surface morphology seen in this study.

J.S. Greenberger: The different in vitro growth patterns seen in B-CLL (Fig. 2) and HCL (Fig. 6) are striking and the cytoplasmic processes developing on HCL are well illustrated in Figures 7 and 8. Did the cellular processes contain collagen types I through V, Laminen or fibronectin? What is the mechanism of surface spreading in these cells compared to those shown in Figure 2? In the text, the authors indicate that the cells resembled fibroblasts or cultured macrophages and thus the question of cell attachment and which specific components cause attachment is quite relevant.
Authors: Unfortunately, we did not study collagen, laminen or fibronectin in any of our cell suspensions and are thus unable to provide data on the biochemistry of the cell processes. We are therefore not in a position to comment on the molecular basis of surface spreading.

J.S. Greenberger: The difference between the leukemic monoblast shown in Figure 12 and the hairy cells shown in Figure 5 is not so striking except for the cell size. What is the variation between populations of cells in each of these two groups and do some specimens overlap in their morphology between groups?
Authors: This question is quite relevant and as a matter of fact in our earliest publications on hairy cells in 1975 we indeed suggested that they were closer to macrophages than lymphocytes because of their similar surface features as seen by SEM. Some overlap does occur and we and other authors including Gamliel and Golomb have outlined these features in other publications. Gamliel and Golomb described this in more detail in a recent review on HCL (38). Briefly, hairy cells generally show ruffles and microvilli on their surfaces with different combinations of these two types of microprojections expressed on the same cell, while monoblasts rarely show both these features on the same cell. Ruffles are the most frequent surface feature seen on monoblasts.

J.S. Greenberger: The morphology of the monoblasts shown in Figure 14 is commonly seen with cells that are dying or have been exposed to serum-free medium, heat or poor incubator conditions with bad acid pH balance. What is the evidence that these changes are specific to TPA and not induced by other toxic environmental conditions? Clones such as those shown in Figure 15 (prominently adherent B cells, after 6 days culture with TPA), will often happen to a cellular monolayer that is sloughing from the surface of a plastic dish. Is this not a possibility here?
Authors: Based on our extensive experience with SEM over the past 14 years, we do not feel that these features are related to sloughing, cell toxicity or cell death induced by toxic environmental conditions, or poor incubator conditions with bad acid pH balance. We are aware of the role environmental factors play in determining cell surface alterations but do not feel this is the case in our study. However, specific parallel controlled samples testing this hypothesis were not performed in these studies.

J.S. Greenberger: The authors use the term "plasmacytoid transformation of cells" in the text but it is not clear on what basis this description is being given. Do the authors have some evidence of plasma cells that have been purified from normal bone marrow and are these features truly found in plasma cells?
Authors: The plasmacytic features described were readily evident on light microscopy and in particular on transmission electron microscopy (TEM). Cells showed as eccentric nucleus with abundant basophilic cytoplasm and prominent rough endoplasmic reticulum was evident on TEM. Furthermore the induction of tartrate-sensitive acid phosphatase provided further evidence for this hypothesis while cells secreted immunoglobulin into the culture medium. All the above features are compatible with "plasmacytoid" transformation. These features have been described in our earlier publications and have been noted by different groups studying the effects of TPA on B-CLL cells.

J.S. Greenberger: Attempts to show the increase in the number of cells that are surface marker positive are depicted in Figure 19. Controls for other toxic agents would be of interest including heat, UV irradiation, exposure to alkylating agents or other non-tumor promoter phorbol esters. Were these performed at any time?
Authors: We cannot reply readily to this interesting question as we have not used the above procedures or agents as controls in our studies. Nevertheless, we can state that some of the surface markers for B-cells such as mouse rosetting qualities tend to disappear during in vitro incubation of viable control cells unexposed to TPA.

J.S. Greenberger: The data shown in Figures 20 and 21 (relating to TPA-induced secretion in CLL cells and TPA-induced acid phosphatase in CLL cells) are more convincing evidence of induced differentiation by TPA. It would help to have standard error bars or at least the number of times the experiment was done, to describe the margin of significance in the data in these two figures. Could you comment?
Authors: No standard error bars were determined in these cases, however the experiment (Fig. 20) was done in triplicate samples and the variations were less than 10%. The results depicted in Fig. 21 are based on counting 500 cells in duplicate samples from each case.

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