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Interaction of peanut agglutinin with normal human lymphocytes and with leukemic cells

(lectin/human thymocytes/acute leukemia/chronic leukemia)

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ABSTRACT **The interaction of peanut agglutinin (PNA) with human thymocytes, peripheral blood lymphocytes, and peripheral blood cells of various types of leukemia was investigated by using fluorescein isothiocyanate-conjugated PNA. The majority of human thymocytes (60–80%) bind the lectin. The major subpopulation of thymocytes that is PNA-positive was separated from the PNA-negative cells by differential agglutination with the lectin. The two thymocyte subpopulations were tested in the mixed lymphocyte reaction and with the phytohemagglutinin of *Phaseolus vulgaris*. The poor response of the PNA-positive thymocytes to these stimuli indicates that these thymocytes are functionally immature. The fluorescein isothiocyanate-PNA-binding test with peripheral blood lymphocytes of leukemic patients revealed that in most acute leukemias the PNA receptor is exposed on the blastic cells, whereas in most cases of chronic leukemia the peripheral blood lymphocytes are PNA-negative. The validity of PNA as a marker of immature blood cells and its potential clinical application are discussed.**

Classification of the various leukemias according to the cellular origin of their pathologic cells is of diagnostic and therapeutic importance. However, such a classification is hampered by the scarcity of cell markers that can be attributed to lymphoid or myeloid subpopulations at specific stages of their differentiation (1). It has recently been shown that peanut agglutinin (PNA) binds exclusively to undifferentiated murine lymphocytes such as immature hydrocortisone-sensitive murine thymocytes (2), fetal liver lymphocytes (3), and bone marrow and spleen stem cells (4). On mature lymphocytes the lectin receptor is masked by sialic acid and can be exposed upon treatment of the cells with neuraminidase.

In the present study we have investigated the interaction of PNA with human thymocytes, peripheral blood lymphocytes, and various types of leukemic blast cells. The validity of PNA as a marker of immature human blood cells and its potential for clinical application are discussed.

MATERIALS AND METHODS

PNA was purified by affinity chromatography on a column of Sepharose-*N*-(ε-aminocaproyl)-β-D-galactopyranosylamine (5). Fluorescein isothiocyanate (FITC) was conjugated with PNA as described (6), and the conjugated lectin was repurified by affinity chromatography. ^{125}I-labeled PNA (^{125}I-PNA) was prepared as described (2), and the iodinated lectin was purified on a Sephadex G-150 column followed by affinity chromatography. Phytohemagglutinin (PHA) was obtained from Wellcome–Burroughs. [*methyl*-^{3}H]Thymidine (5 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was obtained from the Nuclear Research Center, Negev, Israel. Neuraminidase of *Vibrio cholera* was obtained from Behringwerke, Marburg, Germany; 1 unit is defined as the amount of enzyme that liberates 1 μg of *N*-acetylneuraminic acid from α_1-glycoprotein within 15 min at pH 5.5 and 37°C.

Cell Preparations. Normal peripheral blood lymphocytes were purified from heparinized peripheral blood by centrifugation over Ficoll/Hypaque (specific gravity 1.078) (7). Normal human thymuses were obtained from children (aged 2–10 years) undergoing cardiac surgery, in which partial thymic resection is occasionally necessary to facilitate surgical exposure. A single cell suspension of thymocytes was prepared by mincing the thymus in phosphate-buffered saline pH 7.4 (P_i/NaCl), followed by filtration through a fine stainless steel mesh. Leukemic cells obtained from peripheral blood were processed exactly as normal peripheral blood lymphocytes were.

Preparation of Macrophage Monolayers. Aliquots (0.5 ml) of Ficoll-separated mononuclear cells, containing 1–2 × 10^5 monocytes, were incubated for 2 hr at 37°C in tissue culture tubes (16 × 125 mm, Falcon 3033). The nonadhering cells were removed by repeated washings with P_i/NaCl.

Mitogenic Stimulation. Thymocytes (1 × 10^6 per ml) were cultured in RPMI 1640 medium supplemented with 10% human AB serum and 5 μg of PHA. Cultures (1 ml) were prepared in triplicate in loosely capped polystyrene tubes (17 × 100 mm) and incubated at 37°C in an atmosphere of 95% air/5% CO_2 for 72 hr. For the last 2 hr of culture, the cells were pulsed with 1.25 μCi of [^{3}H]thymidine. The incorporation of radioactivity into DNA was determined (8). In the experiments designed to test the effect of macrophages on thymocyte stimulation, the thymocytes (1 × 10^6) were added to tubes layered by macrophages as described above.

Mixed Lymphocyte Culture. Lymphocytes (1 × 10^6 cells) were cultured in Cooke round-bottom microtiter plates together with allogeneic peripheral blood lymphocytes (1 × 10^6 cells) that had been irradiated with a dose of 1500 rads (15 grays). The culture medium consisted of 150 μl of RPMI 1640 buffered with bicarbonate and containing penicillin (100 units/ml), streptomycin (100 μg/ml), and mercaptoethanol (10 μM) and was supplemented with 20% heat-inactivated human AB serum. After 4 days of incubation at 37°C in an atmosphere of 95% air/5% CO_2, the cells were pulsed with 0.4 μCi of [^{3}H]thymidine. After a further 2 hr of incubation, the cells were harvested by the multiple cell harvester (Tritertek Microtitration Equipment, Flow Laboratories, Rockville, MD), and thymidine incorporation into DNA was determined (8).

FITC-PNA Binding Assay. Cells (0.1 ml, 3–5 × 10^6) were incubated with FITC-PNA (0.1 ml, 500 μg/ml) for 15 min at room temperature. The cells were then washed three times with

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Abbreviations: PNA, peanut agglutinin; FITC, fluorescein isothiocyanate; PHA, phytohemagglutinin of *Phaseolus vulgaris;* P_i/NaCl, phosphate-buffered saline (0.15 M NaCl, 0.05 M sodium phosphate, pH 7.4); E rosette, erythrocyte rosette.

P_i/NaCl and the pellet was resuspended in 0.1 ml of P_i/NaCl. The percentage of fluorescent cells was determined for 200 cells counted. Two controls were carried out for each individual assay: (*i*) inhibition by 0.2 M D-galactose (the sugar inhibitor of PNA); (*ii*) pretreatment of the cells with neuraminidase, 50 units/ml at 37°C for 30 min, followed by washing of the cells (three times with 5 ml each of P_i/NaCl).

Binding of Iodinated PNA to Human Thymocytes. Thymocytes (5×10^7 cells per ml) were suspended in 0.2 ml of P_i/NaCl containing 0.1 mg of bovine serum albumin (to reduce nonspecific binding) and were incubated for 60 min at room temperature with different concentrations of the iodinated lectin. The incubation was carried out in Beckman microtubes containing 100 μl of an oil mixture [di-(1-butyl)phthalate/di-(1-octyl)phthalate, 1:1 vol/vol]. Because the tubes are very narrow, the reaction solution could be layered above the oil phase with an air bubble separating them, and there was no contact between the reaction solution and the oil phase during incubation. After incubation the tubes were centrifuged at 11,600 rpm in a Beckman Microfuge for 30 sec. The tubes were cut above the sedimented cell pellet and both the pellet and the supernatant were assayed for radioactivity in a Packard gamma counter. The amount of iodinated lectin bound specifically was calculated by subtracting the amount of iodinated lectin bound in the presence of its inhibitor (0.2 M D-galactose) from that bound in its absence. The nonspecific binding did not exceed 10% of the total binding. All experiments were carried out in triplicate, and the results given are the average values; the individual results did not differ by more than 10%.

Separation of Normal Human Thymocytes into Two Subpopulations by Agglutination with PNA. The thymocyte suspension (0.25 ml, 4×10^8/ml) was incubated in a 5-ml plastic tube with PNA (0.25 ml, 2 mg/ml of P_i/NaCl) for 15 min at room temperature. At the end of the incubation the cells were layered gently with a pasteur pipette on the top of heat-inactivated fetal calf serum (50%, 8 ml) in a conical 12-ml glass tube. After 20 min at room temperature most of the thymocytes sedimented, whereas the unagglutinated single cells remained on the top. The bottom and top fractions (approximately 1.5 ml each) were removed separately by pasteur pipettes and transferred into 10-ml plastic tubes, and the total volume of the cell suspension in each tube was brought up to 10 ml by the addition of galactose solution (0.2 M D-galactose in P_i/NaCl). The cells were collected by centrifugation ($400 \times g$, 10 min); the pellets were washed with D-galactose to dissociate the agglutinated cells, centrifuged again, and resuspended in the galactose solution. Finally the cells were washed twice with P_i/NaCl. A trypan blue exclusion test showed that both fractions contained 90–95% viable cells. In a control sample treated with P_i/NaCl instead of PNA, all the cells remained on top of the fetal calf serum solution, thus eliminating the possibility that the separation involved other factors, apart from agglutinability, such as size or density of the cells.

RESULTS

Interaction of PNA with Normal Human Peripheral Blood Lymphocytes. Normal peripheral blood lymphocytes do not bind PNA unless treated with neuraminidase. The peripheral blood lymphocytes of 20 donors were tested for FITC-PNA binding, and it was found in all cases that less than 2% of lymphocytes bound the lectin. Upon removal of sialic acid by neuraminidase, 100% of the cells bound FITC-PNA.

Separation of Normal Human Thymocytes into Two Subpopulations by Agglutination with PNA. Unlike peripheral blood lymphocytes, the majority of human thymocytes bind FITC-PNA (60–80% of cells). By differential agglutination with PNA, two functionally distinct thymocyte subpopulations were obtained. The subpopulation that is agglutinated by PNA was found to be inactive in the mixed lymphocyte culture reaction and did not respond to PHA, whereas the minor subpopulation that is not agglutinated by PNA does exhibit these responses, similar to peripheral blood T cells (Fig. 1).

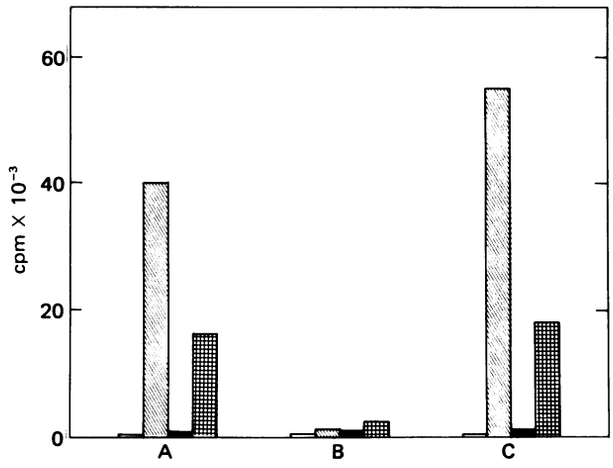

FIG. 1. The mitogenic response ([^{3}H]thymidine uptake) to PHA and in the mixed lymphocyte culture of human thymocytes. (*A*) Unseparated thymocytes; (*B*) thymocytes agglutinated by PNA; (*C*) thymocytes unagglutinated by PNA. Empty bars, thymocytes without PHA; diagonally hatched bars, thymocytes with PHA; solid bars, thymocytes with irradiated syngeneic thymocytes; cross-hatched bars, thymocytes with irradiated allogeneic peripheral blood lymphocytes.

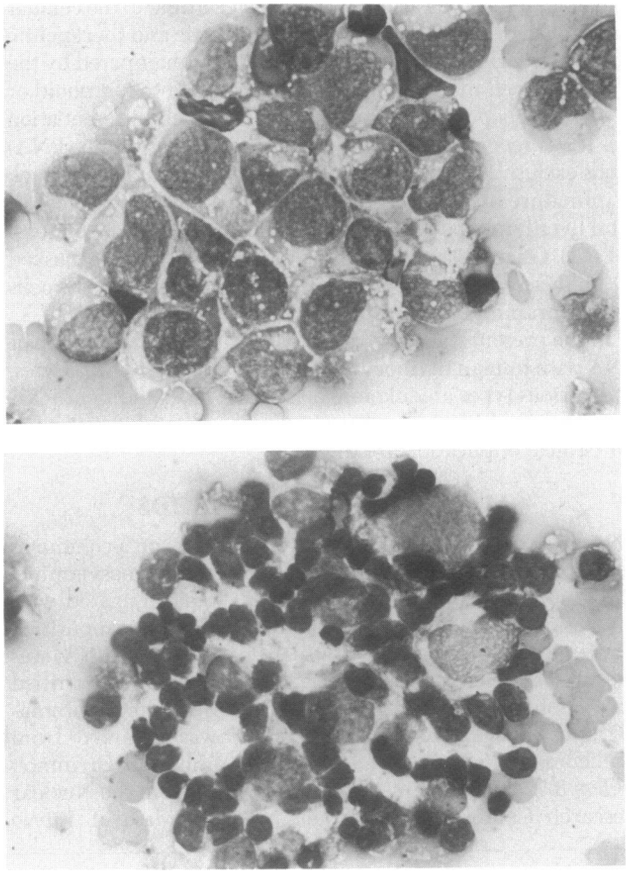

FIG. 2. Giemsa staining of thymocytes after 68-hr incubation with PHA. (×700.) (*Upper*) Thymocytes unagglutinated by PNA; (*Lower*) thymocytes agglutinated by PNA.

Table 1. Effect of macrophages on the mitogenic response to PHA of separated thymocytes

Exp.	Thymocyte fraction	[^{3}H]Thymidine incorporation, cpm per 1 × 10^6 cells: Thymocytes, Control	Thymocytes, With PHA*	Macrophages† + thymocytes, Control	Macrophages† + thymocytes, With PHA*
I	Unseparated	1150	4,300	950	65,000
	Bottom	650	2,450	650	11,500
	Top	1250	22,900	1150	148,000
II	Unseparated	1400	10,500	900	18,000
	Bottom	700	5,000	950	9,200
	Top	1600	11,000	1600	14,500
III	Unseparated	670	34,000	1250	29,500
	Bottom	950	1,450	650	11,500
	Top	1100	31,000	1900	83,000
IV	Unseparated	500	10,500	370	11,000
	Bottom	850	1,850	480	2,000
	Top	520	12,500	620	18,000

* PHA was added at 4 μg/ml.
† Macrophages alone are not stimulated.

Addition of monocytes enhanced the mitogenic response to PHA of both fractions. However, the difference in extent of the response between the two thymocyte subpopulations persisted (Table 1). Giemsa staining of cells taken after 68 hr culture with PHA revealed marked differences in the extent of blastogenic transformation between the two fractions. Most of the cells in the unagglutinated fraction were stimulated, while very few blasts were observed in the agglutinated fraction (Fig. 2). It seems, therefore, that the poor activity of the agglutinated thymocytes is not due to a selective depletion of monocytes but it rather reflects a characteristic of the immature cells.

In both subpopulations more than 90% of the cells bear the receptor for erythrocyte (E)-rosette formation (9).

The two separated thymocyte fractions were also tested for PNA binding by FITC-PNA and by radioactively labeled ^{125}I-PNA. Binding experiments with FITC-PNA revealed that in the unagglutinated fraction only about 10% of the cells were PNA-positive, whereas the agglutinated fraction consisted 100% of such cells. The average total number of PNA-binding sites per cell in each fraction was estimated with the aid of ^{125}I-PNA. As shown in Fig. 3, a marked difference in the extent of PNA binding to the two thymocyte subpopulations was found. Processing the binding data according to Scatchard (10) (Fig. 4) and assuming a contamination of the top fraction with 5–15% of the agglutinable cells (as indicated by the test with FITC-PNA), we found that the total number of binding sites on the mature thymocytes is (2.5 ± 0.74) × 10^4 per cell, whereas on the agglutinable immature thymocytes, the total number of binding sites is (1.5 ± 0.075) × 10^5 per cell. Thus the cells scored as negative in the FITC-PNA-binding test have significantly fewer binding sites per cell.

Interaction of PNA with Various Types of Leukemic Cells. Pathologic cells of different types of leukemia vary in their PNA-binding properties. In most cases of acute lymphatic leukemia and stem cell leukemia (14/20), and myeloid leukemia (5/6), the blasts were PNA-positive in the FITC-PNA-binding test (Table 2). Four patients with Burkitt tumor and one patient with IgG-positive acute leukemia were tested and were also found to be PNA-positive (Table 2). On the other hand, out of 32 patients with chronic lymphatic leukemia, we found PNA-positive cells in only 3, all of whom had low and stable leukocyte counts.

DISCUSSION

PNA interacts preferably with D-galactosyl-β(1→3)-*N*-acetyl-D-galactosamine, although it can also interact with D-galactosyl-β(1→3)-*N*-acetyl-D-glucosamine (5). On most cell membranes these sugar sequences are terminated by sialic acid. Previous studies have demonstrated that PNA binds to immature mouse thymocytes (2), as well as to hemopoietic stem cells in mouse bone marrow and spleen (4). Mature thymocytes (2)

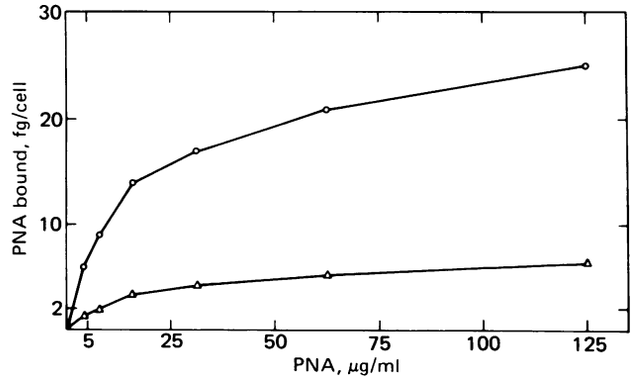

FIG. 3. Binding of ^{125}I-PNA to human thymocyte subpopulations separated by PNA. O, Thymocytes agglutinated by PNA; Δ, thymocytes unagglutinated by PNA.

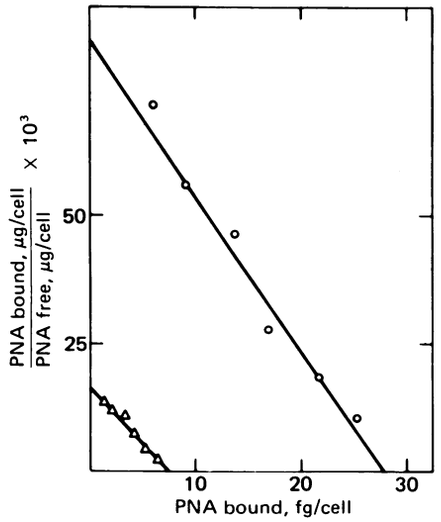

FIG. 4. Scatchard plot of ^{125}I-PNA binding to human thymocytes. O, Thymocytes agglutinated by PNA; Δ, thymocytes unagglutinated by PNA.

Table 2. FITC-PNA binding to various types of acute leukemia* and Burkitt tumor cells

Patient	Peripheral blood† Leukocytes/ mm³	Peripheral blood† % blasts	Ficoll-separated mononuclear cells Ig-bearing cells‡ % IgG	Ficoll-separated mononuclear cells Ig-bearing cells‡ % IgM	% E rosettes‡	% PNA-positive
			Stem cell and acute lymphatic leukemia			
A.M.	100,000	95	<2	<2	2	>80
A.R.	26,000	70	<2	<2	3	<2
S.B.	110,000	99	<2	<2	1	>80
A.S.	ND	70	ND	ND	4	<2
Y.A.	18,000	50	35	40	18	60
K.D.	BM	>80	>80	>80	15	>80
K.Y.	600,000	100	<2	<2	90	>80
O.H.	BM	>80	<2	3	12	>80
K.Y.	20,000	80	ND	ND	5	>80
H.A.	16,000	60	5	4	18	80
A.A.	10,000	60	<2	<2	27	<2
A.H.	300,000	100	6	10	0	80
K.M.	35,000	90	<2	<2	0	>80
G.N.	5,000	40	ND	ND	61	60
J.A.	60,000	90	<2	2	3	>80
B.N.	8,500	70	15	6	2	<2
S.R.	10,600	90	<2	<2	40	<2
B.H.	120,000	80	<2	<2	57	<2
B.B.	30,000	85	<2	5	15	27
A.A.	500,000	100	<2	<2	48	>80
			Myeloid monocytic leukemia			
N.D.	11,000	60	<2	5	13	>80
K.R.	24,000	40	<2	<2	1	>80
N.T.	35,000	80	<2	<2	4	3
L.Z.	7,000	70	ND	ND	11	4
B.A.	3,800	60	8	<2	4	32
S.O.	13,000	70	3	2	3	80
G.E.	150,000	60	30	5	0	>80
			Burkitt tumor			
B.G.	BM	>80	>80	>80	13	30
G.Y.	BM	>80	>80	>80	15	50
M.R.	BM	>80	>80	>80	20	80
G.A.	ST	>80	30	>80	0	80

ND, not determined.

* The classification of leukemias was according to morphologic and cytochemical parameters used in the literature (11, 12).

† Peripheral blood was used except in those cases designated as BM (bone marrow) or ST (solid tumor).

‡ Surface immunoglobulins (2) and E rosettes (9) were tested as described.

or spleen T cells (13) do not bind the lectin unless pretreated with neuraminidase. Therefore, it has been suggested that, during T cell maturation in the mouse thymus, the PNA receptor is being masked by sialic acid (2). Similar results were obtained in the present study on human thymocytes. The PNA-positive thymocytes respond poorly to PHA and in the mixed lymphocyte culture test, whereas the PNA-negative subpopulation responded to both stimuli. Because it has been shown that monocytes can enhance the response of human lymphoctyes to mitogens or in the mixed lymphocyte reaction (7, 14), the poor response of the PNA-agglutinated cells could be attributed to a selective depletion of monocytes from this cell fraction. The results in Table 1 show that the marked difference in the response to PHA between the two subpopulations was not affected by the addition of monocytes to the cell culture.

In all the experiments reported in this paper (except experiment II in Table 1), there was a marked enrichment in the response to PHA of the unagglutinated cells in comparison with that of the unfractionated ones, indicating that the agglutinated cells do not contribute significantly to the total response of the unfractionated cells. Moreover, in our previous work with mouse lymphocytes, we demonstrated that agglutination with PNA and soybean agglutinin and subsequent dispersion of the agglutinates with D-galactose yielded cells that were functional in a variety of biological tests, both *in vitro* (2, 13) and *in vivo* (4, 15). It is thus highly improbable that the lack of response of the agglutinated human thymocytes to PHA is the result of cell damage caused by agglutination. Therefore the PNA-positive human thymocytes are functionally immature. In the mouse thymus, the immature subpopulation is sensitive to hydrocortisone treatment and can be selectively eliminated. Because no such manipulations can be performed in humans, PNA provides a useful tool for the separation of the mature human thymocytes from the immature ones.

The finding that more than 90% of the immature PNA-positive thymocytes bear the receptor for E-rosette formation suggests that the masking of the PNA receptor by sialic acid occurs at a later stage of T cell maturation than the expression of the E-rosette receptor. PNA is thus a more sensitive marker of T cell immaturity than E rosette formation.

Because in normal peripheral blood lymphocytes the percentage of PNA-positive cells is less than 2%, even a minor in-

crease in the number of such cells can be easily detected. Such an increase could be an indication of cell immaturity, and therefore the FITC-PNA-binding test carries a considerable diagnostic potential. Combined with other diagnostic methods, it could, for example, be of aid in the examination of various immunologic disorders. It may be especially useful in cases in which thymic hormone therapy is considered.

It is well known that leukemic cells vary in their degree of differentiation. For example, in chronic lymphocytic leukemia the pathologic cells are relatively mature lymphocytes, while in the acute leukemias immature cells accumulate. It was therefore of interest to compare the binding of PNA to the various types of leukemic cells (Table 2). We found that the lymphocytes of the majority of chronic lymphocytic leukemia patients tested did not bind the lectin, and in this respect were similar to normal peripheral blood lymphocytes (which are mature), while PNA did bind to the blastic cells in most cases of acute leukemia, as well as to cells of Burkitt lymphoma involving the bone marrow.

Assuming that a correlation exists between the properties of leukemia cells and those of their normal progenitor cells, the presence of PNA receptors on the blasts in most cases of acute leukemia suggests that the PNA receptor is exposed on the primitive blood stem cells. This suggestion is in accordance with our recent finding that PNA-positive cells from mouse bone marrow and spleen are capable of forming spleen colonies and of reconstituting lethally irradiated allogeneic mice (4). Our present data indicate that the PNA receptor is masked by sialic acid at a relatively late stage of T cell maturation in the human thymus. If indeed this receptor is exposed on the pluripotential blood stem cell, it is possible that this receptor is also exposed on prethymocytes, which are supposed to represent an intermediate stage between the pluripotential stem cell and the immature thymocytes.

The cases of acute leukemia in which we did not find PNA-positive blast cells may reflect other pathways of differentiation, where masking of the PNA receptors may occur at an earlier stage. Studies on PNA binding to normal immature subpopulations of the lymphoid and myeloid series may clarify this question; however, the low number of such cells in the various lymphoid organs is a major obstacle for studies of this kind.

The immaturity of the blasts is only one possible explanation for the observed results in the FITC-PNA-binding test. The fact that in two cases of T cell acute leukemia (E-rosette-positive) the blasts did not bind FITC-PNA and in a few other cases the percentage of blasts that bound FITC-PNA was significantly lower than the total percentage of blastic cells could indicate membrane defects in such cells with a loss or shortening of galactoglycolipids or galactoglycopeptides. Such changes have been found in the past in various transformed cell lines (16).

The presence of PNA receptors on the blastic cells of many patients with acute leukemia could be of considerable importance because they may serve as targets for drug binding and enable a selective destruction of pathologic cells. This may be particularly useful in cases in which the normal bone marrow is severely suppressed due to therapy while blastic cells persist. PNA, if bound to a drug, may direct and concentrate the drug on this pathologic subpopulation. The use of daunomycin bound to concanavalin A has been demonstrated in a mouse model system (17). The advantage of PNA over other lectins is that it does not interact significantly with human erythrocytes (5) or with normal peripheral blood lymphocytes.

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