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Peanut agglutinin: a marker for normal and leukaemic cells of the monocyte lineage

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SUMMARY

The potential of peanut agglutinin (PNA) as a monocyte lineage marker and a diagnostic tool was investigated. Peripheral blood mononuclear cells (MNC) from normal adults, and 20 patients with acute non-lymphocytic leukaemia (ANLL), as well as five human cell lines were analysed for fluorescein isothiocyanate (FITC)-PNA binding using the fluorescence activated cell sorter (FACS). Monocytes and monoblasts, which were positive for non-specific esterase activity, bound FITC-PNA. In addition, FITC-PNA was bound by a population of cells not defined by cytochemical criteria which were probably precursors to monoblasts. The classical myeloid blast cell did not bind FITC-PNA. ANLL classification is discussed in the light of these results. FITC-PNA clearly binds to cells of the monocyte lineage, and has potential as an adjunct to current cytochemical and morphological criteria in classification.

INTRODUCTION

The lectin peanut agglutinin (PNA) binds to non-reducing terminal D-galactosyl residues (Lotan *et al.*, 1975). Cell membrane reactivity to PNA has been reported for the less differentiated cells in mouse thymocyte, bone marrow and spleen populations (London, Berrih & Bach, 1978; Reisner *et al.*, 1978) for human thymocytes and some malignant lymphoid cells (London *et al.*, 1979; Ballet *et al.*, 1980; Galili *et al.*, 1981). PNA has therefore come to be regarded primarily as a marker for immature T Cells, and of potential use in identifying malignant cells of the T Cell lineage (Galili *et al.*, 1981).

However, PNA has also been reported to bind to monocytes in the normal human peripheral blood mononuclear cell (MNC) population (London *et al.*, 1979), to granulocytes (Taub, Baker & Madyastha, 1980) and to MNC from a small proportion of patients with acute non-lymphocytic leukaemia (ANLL) (Galili *et al.*, 1981; Reisner *et al.*, 1979). The purpose of this study was to determine whether PNA detected a definable subgroup of ANLL patients, and might therefore be useful in classification of ANLL. It has been found that PNA binds to cells displaying monocytic differentiation but not to myeloblasts, and it may therefore be useful in distinguishing between myeloid and myelomonocytic leukaemia.

MATERIALS AND METHODS

Mononuclear cells (MNC) were prepared from heparinized (12·5 units/ml) peripheral blood of normal adult volunteers, patients with newly diagnosed leukaemia and patients in leukaemic

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relapse. The MNC fraction was separated by buoyant density centrifugation using Sepalymph (Teva Pharmaceuticals, Jerusalem) and washed three times in RPMI 1640 (GIBCO, New York, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) and penicillin–streptomycin (100 μg/ml) (Commonwealth Serum Laboratories, Melbourne). The MNC were resuspended to 1×10^7 cells/ml in 10% dimethyl sulphoxide (DMSO) 20% FCS in RPMI 1640, controlled-rate frozen and stored in liquid nitrogen until required.

The human cell lines HL-60, U937, K562 were kindly provided by Dr David Jose, Clinical Immunology and Immunogenetics Unit, The Cancer Institute, Melbourne. The human cell line RC2a was kindly provided by Dr Ray Bradley, The Cancer Institute, Melbourne.

Cell isolation procedures. Normal MNC (1×10^7 in 10 ml RPMI/10% FCS) were incubated for 1 hr at 37°C, in 5% CO_2 in tissue culture petri dishes. Non-adherent cells were removed by washing and adherent cells were removed by vigourous pipetting. Adherent cells were washed twice in phosphate buffered saline (PBS) with 5% FCS and 0·02% sodium azide and resuspended in the same buffer to 2×10^7 cells/ml for lectin binding.

E-Rosetting (E-RFC) and non-E-rosetting (non-E-RFC) populations were prepared according to the method of Madsen *et al.* (1980). Briefly, MNC were suspended at 2×10^6 cells/ml in RPMI 1640 supplemented with FCS which had been absorbed with sheep red blood cells (SRBC). Equal volumes of MNC and 0·5% aminoethyl-isothiuronium bromide treated SRBC (AET-SRBC) in RPMI/10% FCS were incubated at 37°C for 15 min, centrifuged at 50 *g* for 5 min and incubated at 4°C for 1 hr. The E-RFC and non-E-RFC were separated by buoyant density centrifugation using Sepalymph and the SRBC lysed using 0·8% ammonium chloride in Tris buffer pH 7·6 at room temperature for 5 min. The two cell fractions were washed three times in PBS/5% FCS with 0·02% sodium azide and resuspended in the same buffer to 2×10^7 cells/ml for lectin binding.

All cell lines were grown in RPMI/10% FCS in continuous liquid culture at 37°C in an humidified 5% CO_2 atmosphere. These cells were washed in PBS/5% FCS and 0·02% sodium azide and resuspended to 2×10^7 cells/ml for lectin binding.

Frozen mononuclear cells were rapidly thawed at 37°C and the DMSO was diluted by dropwise addition, mixing and equilibration of RPMI/10% FCS over 45 min. The cells were washed three times in PBS/5% FCS with 0·02% sodium azide and resuspended to 2×10^7 cells/ml for lectin binding.

Viability. Cell viability was assessed by trypan blue exclusion and was greater than 90% for most samples. However, two leukaemia samples, stored for over 2 years had viabilities of 76% and 60%. The dead cells were excluded in fluorescence analysis.

Differential morphology. Cytocentrifuge smears were made of all samples before lectin binding and the differential counts determined using Giemsa stain.

Cytochemical stains. Non-specific esterase and chloracetate esterase stains were performed on cytocentrifuge samples of peripheral blood MNC by the Haematology Division, Institute of Medical and Veterinary Science, Adelaide (Ansley, Sweetman & Ornstein, 1971; Li, Lam & Yam, 1973).

PNA purification. Crude PNA in PBS was prepared using the method of Irle (1977). The crude PNA solution was concentrated to 50 ml by membrane filtration using an Amicon UM10 membrane, 5 ml aliquots were applied to a 5 ml column of neuraminidase treated Fetuin–Sepharose, incubated for 30 min at room temperature and eluted with 100 ml of PBS. Five millilitres of 0·5 M galactose in PBS was then applied to the column, incubated at room temperature for 30 min and eluted with 50 ml of the same buffer. The fractions eluted with galactose were pooled, concentrated and washed with PBS using Amicon filtration. The protein concentration was adjusted to 1 mg/ml and the eluate was analysed under reducing conditions by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) (Achtman *et al.*, 1978). A single band of approximately 30,000 daltons was stained by Coomassie blue.

Fluorescein isothiocyanate (FITC) labelling of PNA. Labelling of PNA was performed using the FITC-celite method of Rinderknecht (1962). The reaction was terminated and free FITC removed by passage of the reaction mixture over a Sephadex G-25 column equilibrated with PBS containing 0·005% polyethylene glycol 6000 and 0·02% sodium azide. Labelled PNA eluted in the void volume, whereas free FITC bound to the gel. The FITC-PNA OD_{495}/OD_{280} ratio was $0{\cdot}98 \pm 0{\cdot}05$ and the

concentration was adjusted to 1 mg/ml using an Amicon centriflo membrane (25,000 molecular weight cut off).

FITC-PNA labelling of cells. FITC-PNA (10–200 μg) was added to 2×10^6 viable cells in 0·1 ml PBS/5% FCS + 0·02% sodium azide or in 0·5 M galactose/PBS/5% FCS and 0·02% sodium azide. The cells were incubated at 4°C for 30 min, washed three times in the same buffer without galactose and resuspended to 0·2 ml. Samples were incubated at 4°C for 1 hr until analysed using the fluorescence activated cell sorter (FACS). Under these conditions no agglutination of cells occurred.

Phagocytosis. Cells were incubated in RPMI/20% FCS with 1 mg/ml Zymosan for 45 min at 37°C and washed 3 times in PBS/5% FCS with 0·02% sodium azide. Cells were then incubated with FITC-PNA as described for FITC-PNA staining. Individual cells were recorded for zymosan ingestion by phase contrast microscopy and for FITC-PNA labelling by flourescence microscopy.

Analysis using the FACS. A Becton Dickinson FACS IV instrument was used with a tunable Argon ion laser (Spectra Physics model 164-05). The laser was operated at the 488 nm emission line at 0·5 watts and a 530 nm band pass filter (8 nm width) was used to detect FITC flourescence. Light scattering intensities were also measured at the 488 nm line with a dichroic mirror inserted (which reflects up to 580 nm) and one neutral density filter (density 3·0). The intensity of the scattered laser light was measured independently in the forward (0° ± 12°C) and side (90° ± 20°) directions relative to the incident beam. Two directional scatter analysis of normal human peripheral blood populations has been shown to give reproducible groupings of cells according to light scatter characteristics (Salzman *et al.*, 1975; Shapiro, 1977). The method used in this study was to observe the light scatter characteristics using two directional scatter, then to select particular groups of cells, based on 0° and 90° parameters, and observe the third parameter, i.e. fluorescence intensity for these individual cell types, as well as fluorescence profiles for the whole sample.

Fluorescence profiles were recorded only for viable nucleated cells, this being taken as cells with 0° scatter intensity greater than channel 80, and 50,000 cells were analysed per sample. Non-fluorescent cells were determined as those cells with a fluorescence intensity equal to or less than the value found for the cells labelled in 0·5 M galactose. This value was determined each day the FACS was used, and repeated several times during each run of samples.

Results are expressed as the percentage of cells in the total population with a fluorescence intensity greater than the control cells.

RESULTS

FITC-PNA binding to normal MNC

Normal MNC fractions from peripheral blood were analysed for FITC-PNA binding at a lectin concentration of 50 μg/ml (Table 1). Two directional scatter analysis (Figs. 1, 2) and differential counts of these mixed populations indicated that cells with higher 0° and 90° scatter intensities were binding FITC-PNA, and that these cells were monocytes (Salzman *et al.*, 1975; Shapiro, 1977).

Table 1. Percentage FITC-PNA binding and differential morphology in normal human MNC populations fractionated by adherence to plastic and E-rosette buoyant density centrifugation

MNC fraction	Differential morphology		% FITC-PNA
	Lymphocytes	Monocytes	
Unfractionated MNC	92±2	8±4	7±3
Adherent MNC	17±2	83±2	84±3
E-RFC	98±2	2±2	1±1
Non-E-RFC	80±5	20±4	22±3

Results are expressed as mean ± s.d. from three samples.

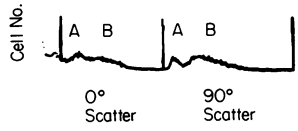


Fig. 1. 0° and 90° light scatter analysis of MNC sample containing 19% lymphocytes and 81% monocytes by differential morphology. Peaks A and B contained 19% and 81% of sample respectivity in the 0° and the 90° light scatter analysis.

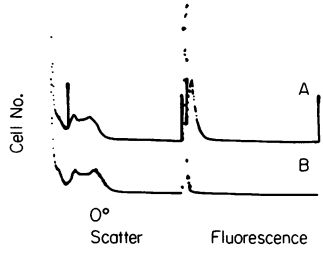
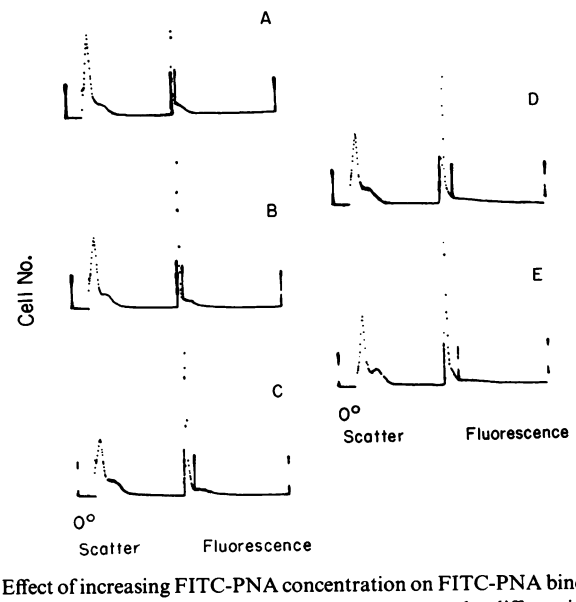


Fig. 2. Effect of 0·5 M galactose on FITC-PNA binding in MNC sample containing 19% lymphocytes and 81% monocytes. (A) MNC + FITC-PNA; (B) MNC + 0·5 M galactose + FITC-PNA.

The freezing and thawing procedure did not alter the percentage of cells binding FITC-PNA (data not shown).

The specificity of the FITC-PNA binding was demonstrated by reacting the FITC-PNA and MNC in the presence of 0·5 M galactose. FITC-PNA binding was completely inhibited in the presence of 0·5 M galactose (Fig. 2).

The non-E-RFC fraction was used to test the effect of FITC-PNA concentration on the percentage of cells binding the lectin and on the amount of lectin bound per cell. Fig. 3 demonstrates that although the lectin concentration was varied over a 20-fold range, the percentage of cells in the



Sample	FITC-PNA (μg/mL)	% FITC-PNA
A	10	14
B	20	16
C	50	19
D	100	19
E	200	20

Fig. 3. Effect of increasing FITC-PNA concentration on FITC-PNA binding and flourescence intensity in MNC sample containing 82% lymphocytes and 18% monocytes by differential morphology.

Table 2. Differential morphology, percentage FITC-PNA binding and initial diagnosis grouping of peripheral blood MNC from 20 patients with ANLL. F.A.B. classifications performed on bone marrow samples from the same patients are included for comparison

	Differential morphology					F.A.B. classification	
MNC sample	% Lymphocytes	% Monocytes	% Blast cells	% FITC-PNA	Initial diagnosis	(1)	(2)
1	6	0	94	1	Adult	M1	M1
2	15	1	84	1	AML	M2	M2
3	5	10	85	5		M4	M4
4	11	8	81	8		M1	M2
5	8	0	92	1		M2	M2
6	3	1	96	1		M1	M1
7a	70	10	20	7		M1	M1
7b	38	4	58	2			
8a	35	41	24	64	Adult	M4	M4
8b	9	7	84	12	AMML		
9	12	3	85	10		M5	M5
10	6	3	91	2		M5	M4
11	13	8	79	94		M5	M5
12	4	63	33	92		M2 (blast crisis)	M2
13	13	85	2	86	Adult	CMML	CMML
14	18	82	0	86	CMML	CMML	M4 Type
15	18	38	44	30		M1 (blast crisis)	CMML
16	15	40	45	38		(M1) (myelofibrosis with blastic change)	(M1)
17	5	92	3	89		CMML	CMML
18	2	2	96	1	Childhood		
19	23	1	76	1	AML	Not tested	
20	12	2	86	96	Childhood mono L	Not tested	

population binding the FITC-PNA became constant, and the fluorescence intensity increased approximately two-fold. Two directional scatter analysis showed that despite lectin concentration changes, the same population of cells was binding FITC-PNA.

PNA-FITC binding to leukaemic cell samples

MNC from 20 patients with various classifications of ANLL were analysed for FITC-PNA binding (Table 2). Patient samples were initially grouped as AML, AMML or CMML, according to a general diagnosis based on peripheral blood picture, cytochemical results and other clinical findings. For comparison, Franco-American-British (F.A.B.) classification (Bennet *et al.*, 1976) was performed blind on patient bone marrow samples by two independent observers.

MNC from seven patients with AML were analysed. Samples 7a and 7b were from the same patient, 7 days apart when the patient was in relapse and untreated. Comparison of the light scattering characteristics and differential morphology with the percentage of cells positive for FITC-PNA suggests that the monocyte population is binding the FITC-PNA. Analysis of the data revealed that the Spearman rank correlation co-efficient between monocyte percentages and FITC-PNA percentages was highly significant ($r_s = 0{\cdot}875$, $P < 0{\cdot}01$). The F.A.B. classification on bone marrow of patient 3 did not agree with the initial diagnosis.

MNC from five patients classified as AMML were analysed for FITC-PNA binding. Samples 8a and 8b were from the same patient at initial diagnosis and first relapse respectively. Samples 8b, 9 and 10 contained both low numbers of monocytes by differential morphology and low percentages of cells binding FITC-PNA, however, no correlation was found between monocyte percentages and FITC-PNA binding in the group as a whole. Fig. 4 demonstrates the FACS results for Sample 12 showing clearly in the scatter analysis that the cells exhibiting higher fluorescence intensity, also exhibited higher 0° light scattering characteristics. The bone marrow from this patient was classified as M2 morphologically, but the peripheral blood picture was that of AMML, with 75% of MNC positive for non-specific esterase.

Cytochemical analysis of these populations by non-specific esterase and chloracetate esterase are shown in Table 3.

Staining results demonstrate that samples 8a, 11 and 12 contain blast cells which have monocytoid cytochemical features, while most blast cells from samples 8b, 9 and 10 have neither monocytoid or myeloid staining characteristics. Lymphocytes in each sample are included in the weakly staining group, but by taking this into account, there is a large population of cells in samples 8b–12 which are not clearly defined by these stains. The FITC-PNA is therefore binding to cells which show monocytoid characteristics (samples 8a, 11, 12) but in addition it must be binding to cells which are weak or negative for these stains. It is interesting to note that in samples 8a and 8b the percentage of cells positive for non-specific esterase dropped when the patient relapsed, as did the percentage of cells binding FITC-PNA. Patients 9 and 10 showed low non-specific esterase activity and PNA binding, but were classified on bone marrow morphology as M5 or M4.

MNC from five patients with CMML (Table 2) gave FITC-PNA binding percentages which correlated significantly ($r_s = 0{\cdot}975$, $P = 0{\cdot}05$) with the monocyte percentages in the samples. Light scatter characteristics showed that the blast cell populations were not binding the FITC-PNA. The blast cells in samples 15 and 16 were positive for chloracetate esterase, demonstrating that they were of the myeloid lineage. Fig. 5 shows the FACS results for sample 15. The small proportion of FITC-PNA positive cells are clearly shown in the scatter diagram to have higher 0° light scattering characteristics.

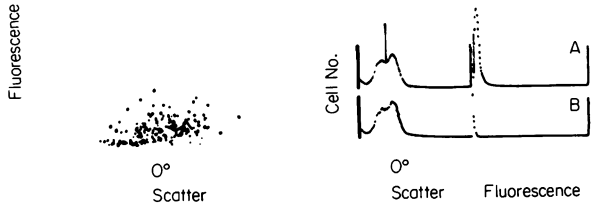


Fig. 4. 0° light scatter and fluorescence analysis on AMML sample 12 which contained 92% FITC-PNA binding cells.

Table 3. Differential morphology, non-specific esterase and chloracetate esterase staining and FITC-PNA binding of MNC from AMML patients

MNC sample	Differential morphology			% Non-specific esterase	% Chloracetate esterase	% Weak or negative	% FITC-PNA
	% Lymphocytes	% Monocytes	% Blasts				
8a	35	41	24	57	0	43	64
8b	9	7	84	7	7	86	12
9	12	3	85	12	2	86	10
10	6	3	91	4	5	91	2
11	13	8	79	23	33	44	94
12	4	63	33	75	2	23	92

Table 4. Non-specific esterase staining, phagocytosis and FITC-PNA binding of human cell lines

Line	Origin	Non-specific esterase	Phagocytosis	% FITC-PNA	Phagocytosis +FITC-PNA
HL-60	Promyelocytic leukaemia	+	+ (10%)	10	+ (10%)
U-937	Histiocytic lymphoma	+	+	1	–
K562	Pleural effusion of CML-blast crisis	–	+	100	–
RC2a	Frozen cells from AMML	+(light)	+	98	Not tested

MNC from two children with AML did not bind FITC-PNA, whereas MNC from patient 20, a child with acute monocytic leukaemia, bound FITC-PNA (Table 2).

FITC-PNA binding to human cell lines

Four human cell lines with some myeloid cell characteristics were analysed for FITC-PNA binding. The myelomonocytic cell line RC2a was highly positive for FITC-PNA, as was the line K562, which has some erythroleukaemia characteristics. However, U-937 with some characteristics of macrophages (Sundstrom & Nilsson, 1976) was negative for FITC-PNA binding and only 10% of the HL-60 cells bound FITC-PNA. This positive fraction was predominantly mature neutrophils, and FITC-PNA labelling of the cells after phagocytosis showed that only those cells capable of phagocytosis were FITC-PNA positive. The remainder of HL-60 cells which, were less differentiated myeloid cells, did not bind FITC-PNA. Fig. 6 shows the results for K562 and HL-60 cells.

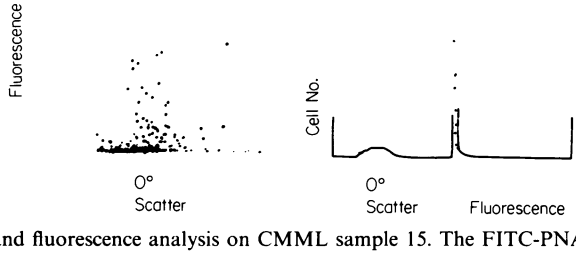


Fig. 5. 0° light scatter and fluorescence analysis on CMML sample 15. The FITC-PNA binding cells (30%) exhibited higher 0° scatter characteristics.

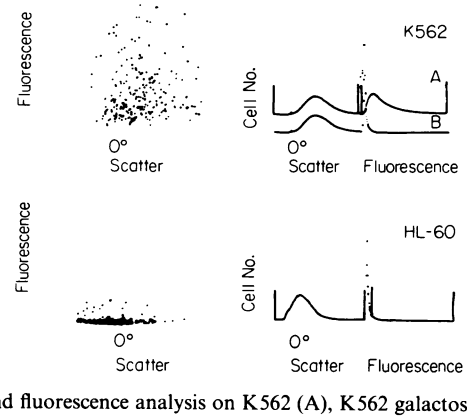


Fig. 6. 0° light scatter and fluorescence analysis on K562 (A), K562 galactose control (B) and HL-60.

DISCUSSION

Many of the proposed classifications of ANLL, do not include immunological markers as in lymphoid leukaemia, but rely on morphology as an absolute standard with confirmation by cytochemical criteria. However, morphological classification can be rendered fairly ineffective by subtle variations within cell lineages. One such widely used classification is the F.A.B. system (Bennet *et al.*, 1976). One of the major difficulties of this system has been the establishment of a clear difference between the M2 and M4 groups; i.e., myeloblastic leukaemia with maturation and myelomonocytic leukaemia. The preferred method of selection is ultimately by cytochemical staining, with the M4 group containing a high proportion of non-specific esterase staining cells, and few cells stained for chloracetate esterase. This method however, also has its pitfalls, since leukaemic myeloblasts and monoblasts often fail to give clear results. Double staining and very light or weak staining can occur, and this is difficult to interpret. Clearly this is an area of classification where alternative techniques should be considered. PNA binding is one technique which has potential as a marker for classification of myelomonocytic leukaemia.

PNA binding demonstrates the presence of non-reducing terminal D-galactose residues in the cell membrane. The results of this study confirm earlier findings that peripheral blood monocytes, but not lymphocytes, bind PNA (London *et al.*, 1979). In addition, it is also evident that peripheral blood monocytes from patients with various forms of non-lymphocytic leukaemia also bind PNA. Of particular interest, however, is that leukaemic monoblasts, and perhaps less differentiated cells in the monocyte lineage, also bind PNA. Galili *et al.* (1981) and Reisner *et al.* (1979) have reported PNA binding to cells from some AMML and AML patients, however, few details of the characteristics of the cells were reported.

Acute myelomonocytic leukaemia is a disease where, the major leukaemic cell type usually shows characteristics of decreasing differentiation as the disease progresses. This is particularly evident when the characteristics of cell types are compared at initial diagnosis and in relapse. The major cell type generally shows a decrease in monocytoid characteristics and a corresponding increase in myeloid characteristics. The decrease in percentage of cells binding PNA shown for patient 8, reflects this change in character of the disease. Perhaps the low percentages of PNA binding (and non-specific esterase activity) in samples 9 and 10 are also an indication of diagnosis at a later stage in the disease. In other samples, cells negative or only weakly stained with non-specific esterase were PNA positive, suggesting that PNA also detects a population of cells in the ill-defined area between the myeloid blast and the monoblast populations. The data from AML patients, both adults and children, clearly shows that the classical myeloblast does not bind PNA, whereas the normal monocytes in the AML samples correlate well with the percentages of cells binding PNA. The blast cell populations from CMML patients were also typical myeloblasts, and the PNA binding in these samples correlated with the monocytes present, and not the blast cells. It is therefore proposed that the PNA binding demonstrated in AMML samples is an indication of monocytes, monoblasts and monoblast precursors in the population, and is of particular use in diagnosis in situations such as that shown for sample 12, where bone marrow F.A.B. classification and the peripheral blood picture are not in agreement, but PNA binding clearly demonstrates that the blast cells in peripheral blood are of the monocyte lineage. In future studies, we intend in addition to analyse PNA binding in bone marrow samples.

The mature or end stage cell of the monocyte lineage is the macrophage, and while there is no macrophage equivalent in the leukaemic populations studied, evidence from the human cell line U-937 (which exhibits many macrophage characteristics) suggests that only the monocyte and its less differentiated form bind PNA. Mature lymphoid cells have been shown to contain a larger amount of sialic acid than immature cells (Despont *et al.*, 1975) and neuraminidase treatment of mature cells is reported to expose PNA binding sites (Dillner-Centerlind *et al.*, 1980). Further studies with macrophages are needed to ascertain if the same phenomenon can be demonstrated in the monocyte lineage.

Recent studies (Galili *et al.*, 1981; Schwenk *et al.*, 1980) have reported that K562 does not bind PNA. However, in both studies, fluorescence microscopy was used for analysis and Schwenk *et al.* (1980) did not include the appropriate galactose inhibition analysis. The detection of FITC-PNA

binding relative to the galactose inhibited control is considerably more sensitive using the FACS, and this is the most likely reason for the difference in results, however there is some debate about K562 having changed characteristics since the original report, and several conflicting reports on its lineage characteristics. The high percentage of K562 cells binding PNA supports the proposal that this cell line is not of the myeloid lineage, but an erythroleukaemia line (Lozzio & Lozzio, 1979; Benz *et al.*, 1980). Studies on samples from erythroleukaemia patients are required to ascertain if this single result has any clinical significance.

Preliminary studies in our laboratory confirm earlier reports (Taub *et al.*, 1980) that granulocytes bind PNA. The PNA binding cells in the HL-60 population exhibited granulocyte characteristics, whereas, the less mature cells of the myeloid lineage, in the same population did not bind PNA. The result reinforces the conclusions drawn from the AML samples, i.e. that the less differentiated cells in the myeloid lineage do not bind PNA.

At present the nature of the PNA binding site is unclear. Its expression on human and murine thymocytes in early stages of maturation, on human monocytes, monoblasts and neutrophils suggest that is may be of importance in differentiation. If there is a role for the PNA binding site, in differentiation, it is clearly different in its expression in each cell lineage.

This diversity of expression however, does not rule out PNA binding as a useful tool. In view of the above data, and the present nebulous nature of myelomonocytic leukaemia classification, PNA is therefore potentially useful in diagnosis and classification of ANLL and in study of monocytic differentiation.

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