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Site-Specific Monoclonal Antibodies Against Peanut Agglutinin (PNA) From Arachis hypogaea

Immunohistochemical Study of Tissue-Cultured Cells and of 27 Cases of Hodgkin's Disease

DANIELA BURRONI, M. CINTORINO,* L. LEONCINI,* P. TOSI,* and C. CECCARINI

*Sclavo Research Center, Siena, and *Institute of Pathological Anatomy, University of Siena, Italy*

The purpose of this study was to increase the sensitivity of the staining reaction for the T antigen on the surface of neoplastic cells grown *in vitro* with the use of site-specific monoclonal antibodies (MAbs). The authors describe anti-peanut agglutinin (PNA) MAbs selected by screening the hybridomas with PNA and PNA bound to bovine serum albumin conjugated with the T antigen. The selected hybridomas (F2C8, F3D12, F3A5) were then grown in pristane-sensitized mice or in the Amicon Hollow Fiber System (F2C8). The affinity constant values for PNA were measured, and all the purified MAbs were tested on both native and denatured PNA, wheat germ agglutinin, concanavalin A, and ricin by using the immunoassay dot test and immunoblotting methods. Eleven different cell lines were stained with the three MAbs; similar results were obtained with F2C8 and F3D12. In each case the fluorescence, if present, was associated with the cell membrane, and the intensity of the staining was always stronger when the cells were incubated with the MAbs than when stained with fluorescein-labeled PNA. On the other hand, F3A5 failed to stain unfixed cells preincubated with PNA but stained the same cells after fixation, independently of the presence of PNA. One of the antibodies, F2C8, was used to stain histologic preparations from 27 cases of Hodgkin's disease and was compared with the anti-granulocyte antibody, Leu-M1, which has been used by numerous authors to identify the characteristic Reed–Sternberg cells. The results obtained were qualitatively similar; ie, F2C8 was at least as efficient as anti-Leu-M1 in its ability to stain the typical diagnostic cells in Hodgkin's disease. (Am J Pathol 1988, 131:351–360)

PLANT LECTINS are a class of proteins capable of binding specific sugar moieties. It is generally accepted that the cell surface contains the majority of the cellular sugar residues, and these lectins have often been used to discriminate between different cell types.[1,2] Another interesting property of certain lectins is that upon binding to the cell surface, they can stimulate cell proliferation.[3,4]

Peanut agglutinin (PNA) is a tetrameric protein isolated from *Arachis hypogaea* capable of binding the disaccharide Galβ1-3GalNAc. This disaccharide is known as the T antigen and can be exposed on red blood cells after treatment with neuraminidase.[5,6] The T antigen has also been shown to be present on the cell surface of a variety of carcinoma cells.[7,8] Several groups of investigators have used fluorescein-labeled PNA or polyclonal antibodies against PNA to identify malignant cells in histologic sections.[9–12] Numerous papers have also appeared in the literature in the past few years that demonstrated that PNA has the ability to discriminate between mature and immature T lymphocytes[13–17] and that it distinguishes between normal blasts and leukemic cells in children with acute lymphoblastic leukemia.[18–19] Other authors reported that PNA has some specificity for Reed–Sternberg (RS) malignant cells in Hodgkin's disease,[20–22] and reports have also appeared indicating well-characterized staining patterns in some cases of histiocytosis X.[23–24]

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Address reprint requests to Dr. Daniela Burroni, Sclavo Research Center, Via Fiorentina 1, 53100 Siena, Italy.

Finally, PNA receptors have been reported as a specific marker for rat brain myelin[25] and for the undifferentiated mouse embryonal carcinoma cell F9.[26]

In this study we describe the property of several monoclonal antibodies (MAbs) raised against PNA. The antibodies in question were selected in a manner that did not interfere with the binding of PNA to its receptor; they were purified to homogeneity and were used to stain a variety of cell types grown in tissue culture. A comparative study of one of these MAbs was also carried out with anti-Leu-M1, an MAb used by numerous authors in investigating Hodgkin's disease.[27-31]

Our data show that staining of the cells with the MAbs against PNA increased the sensitivity of the staining reaction and permitted an easier demonstration of the T antigen on the surface of cells, which was the original aim in undertaking this project. Our comparative study with 27 cases of Hodgkin's disease showed that the MAb selected was at least as efficient as the anti-Leu-M1 in staining the typical diagnostic cells in Hodgkin's disease.

Materials and Methods

Cells and Cell Culture

Two lines of murine myeloma were used for the production of hybridomas: NS1/Ag4 and Sp2/01Ag8. They were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) and 2 mM glutamine. The same medium was used for growing hybridomas with the addition of 10^{-5} M hypoxanthine and 1.6×10^{-5} M thymidine. F9 and PYS were grown in Dulbecco's minimal essential medium (DMEM) plus 10% FBS; Schwann cells, in DMEM plus 20% FBS.[32] S4P77, A549, MKN45, and MKN28 were cultured in 10% FBS–glutamine supplemented with RPMI 1640 and NIH 3T3 HeLa, and HepG2 in 10% FBS–MEM.

Monoclonal Antibodies

BALB/c mice were immunized intraperitoneally and intravenously with PNA (7 injections in 30 days), and the spleen cells were fused with the above mentioned myelomas according to Cianfriglia et al.[33] Screening of the hybridomas was carried out with a radioimmunoassay (RIA) according to the following protocol. Microtiter plates were coated with PNA or PNA immobilized on plates which were initially treated with bovine serum albumin (BSA) modified with covalently linked Galβ1-3GalNAc-β-o-CH2CH-2SCH2CH2CONH-BSA, and each mole of BSA contained 10–20 moles of the disaccharide. The supernatants of all the clones were screened against the above-described substrates with radiolabeled secondary antibody. The hybridomas selected were subcloned three times and expanded by two different methods, ie, in either pristane-sensitized syngeneic mice or in an Amicon Vitafiber II cell culture system.

Purification of MAbs

The ascitic fluid or the culture medium was precipitated with 40% ammonium sulfate, and the precipitated IgG was affinity-purified with a Protein A–Sepharose column according to the method of Ey et al.[34] All the Mabs eluted as IgG1. The result was confirmed by using anti-mouse isotype antibodies.

Amicon Vitafiber II Culture System

The hollow fiber system was preconditioned for 48 hours prior to the introduction of approximately 1.3×10^8 cells. Initially the cells were fed with RPMI 1640 containing 10% HIFBS, and MAbs were not collected during the first week. Starting from the second week, approximately 25 ml of the intra-fiber medium containing the MAbs was collected twice weekly and stored frozen at −30 C. About 5 weeks after the initial inoculation, the concentration of HIFCS was reduced to 5%, to facilitate the purification of the MAb. The medium, 1 liter, was changed daily; and the cartridge was discarded after approximately 12 weeks. When the serum content was kept at 5%, we were able to purify between 100–140 μg of MAb for each ml of medium (approximately 50 ml of culture medium was collected weekly). Lower serum concentration rapidly decreased the yield of MAb.

Affinity Constants

These experiments were carried out using the RIA technique on multiwell plates coated with Galβ1-3GalNAc-BSA plus PNA. The MAbs were labeled with ^{125}I; then, with a constant amount of label, a competition protocol was set up with increasing concentrations of unlabeled MAbs. The results were elaborated with a VAX 11/750 with a program developed by Antoni et al.[35]

Cell Immunostaining

The cells were allowed to adhere to glass coverslips and 2 days after were washed with a 50% mixture of phosphate-buffered saline (PBS) and growth medium lacking FBS and incubated with 0.05 ml of 0.02 mg/

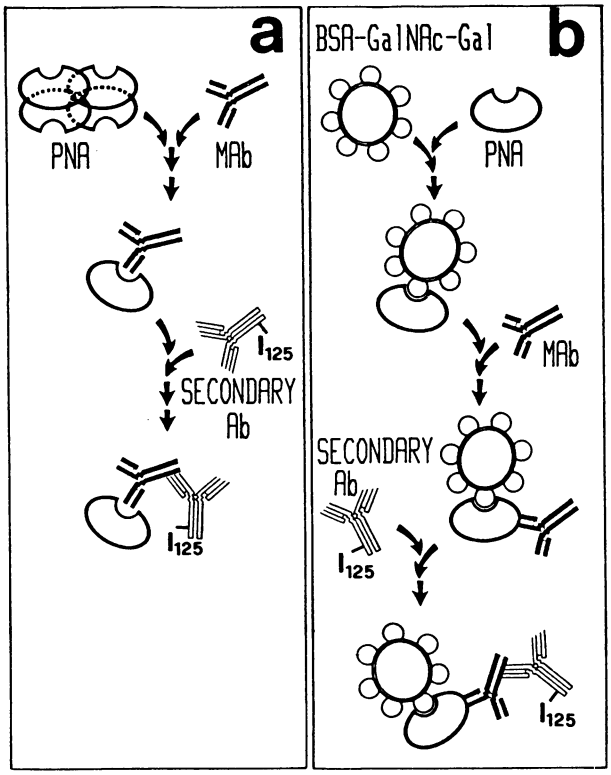

Figure 1—The drawing shows the protocol used to select MAbs against PNA. In protocol **a**, MAbs could also bind to the saccharide-binding site of PNA. In protocol **b** MAbs should not bind to the saccharide-binding site of PNA.

ml of highly purified PNA (Miles) made up with the washing solution for 30 minutes at 37 C or on crushed ice. The coverslips were washed and incubated with MAbs for 30 minutes at 37 C or on ice, washed again, and then stained with a fluorescein isothiocyanate (FITC)-labeled secondary antibody (Southern Biotechnology Associates), goat anti-mouse IgG diluted 1:200. With a similar protocol, cells were also stained with fluorescein-labeled PNA, either purchased from Sigma or prepared in our laboratory according to the method of The and Feltkamp.[36] In this case, the cells were incubated with the labeled lectin, again at a concentration of 0.02 mg/ml. Finally the cells were fixed in acetic acid:ethanol (5:95) for 10 minutes at −20 C. The coverslips were then placed on slides with 0.005 ml of 50% glycerol–PBS and sealed with transparent nail polish. The cells were usually observed and photographed within 48 hours with a Zeiss IM/35 inverted microscope equipped with an epi-fluorescence illuminator, with Kodak Ektachrome 160 ASA film.

Gel Electrophoresis, Immunoblotting, and Dot Blot Assay

The purity of the MAbs was evaluated with polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS and mercaptoethanol (SDS-PAGE) (not shown). Similar gels were used for electrophoresis of lectins. For the immunoblotting studies the proteins were transferred to nitrocellulose sheets at 4 C for 4 hours at 250 milliamperes and revealed by the peroxidase staining method.[37] Staining of native PNA and other lectins was carried out by dispensing known amounts of protein (about 0.5 μg) in 0.5 μl on nitrocellulose paper and using the peroxidase method.

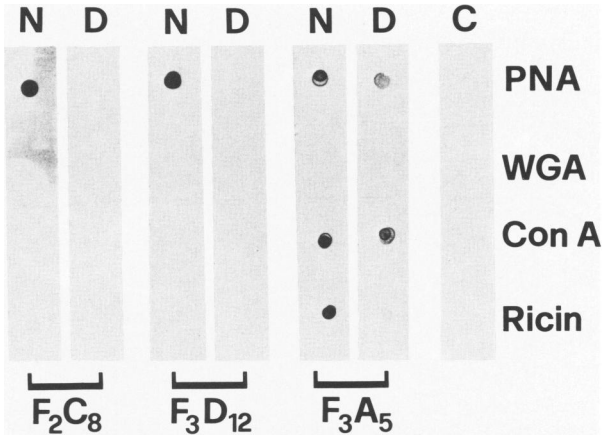

Figure 2—Dot blot test on native (*N*) and denatured (*D*) lectins. The development time was the same for each lane (15 minutes). However, those lectins which were negative remained so even when the development time was increased to 30 minutes.

Studies on 27 Cases of Hodgkin's Disease

All lymph nodes were fixed in 10% formalin and embedded in paraffin, and 4-μ sections were cut and routinely stained with hematoxylin and eosin (H&E), Giemsa, periodic acid–Schiff (PAS), and Gomori. The cases were subdivided according to the Rye classification, as follows: 7 lymphocyte predominance (LP) (5 diffuse and 2 nodular subtypes), 2 mixed cellularity (MC), 2 lymphocyte depletion (LD), and 16 nodular

Table 1—Summary of Immunoassay Dot Tests

	Monoclonal antibodies		
	F2C8	F2D12	F3A5
PNA			
Native	+++	+++	++
Denatured	−	−	++/−
WGA			
Native	−	−	−
Denatured	−	−	−
Con A			
Native	−	−	+++/−
Denatured	−	−	++/−
Ricin			
Native	+/−	+/−	+++/−
Denatured	−	−	+/−

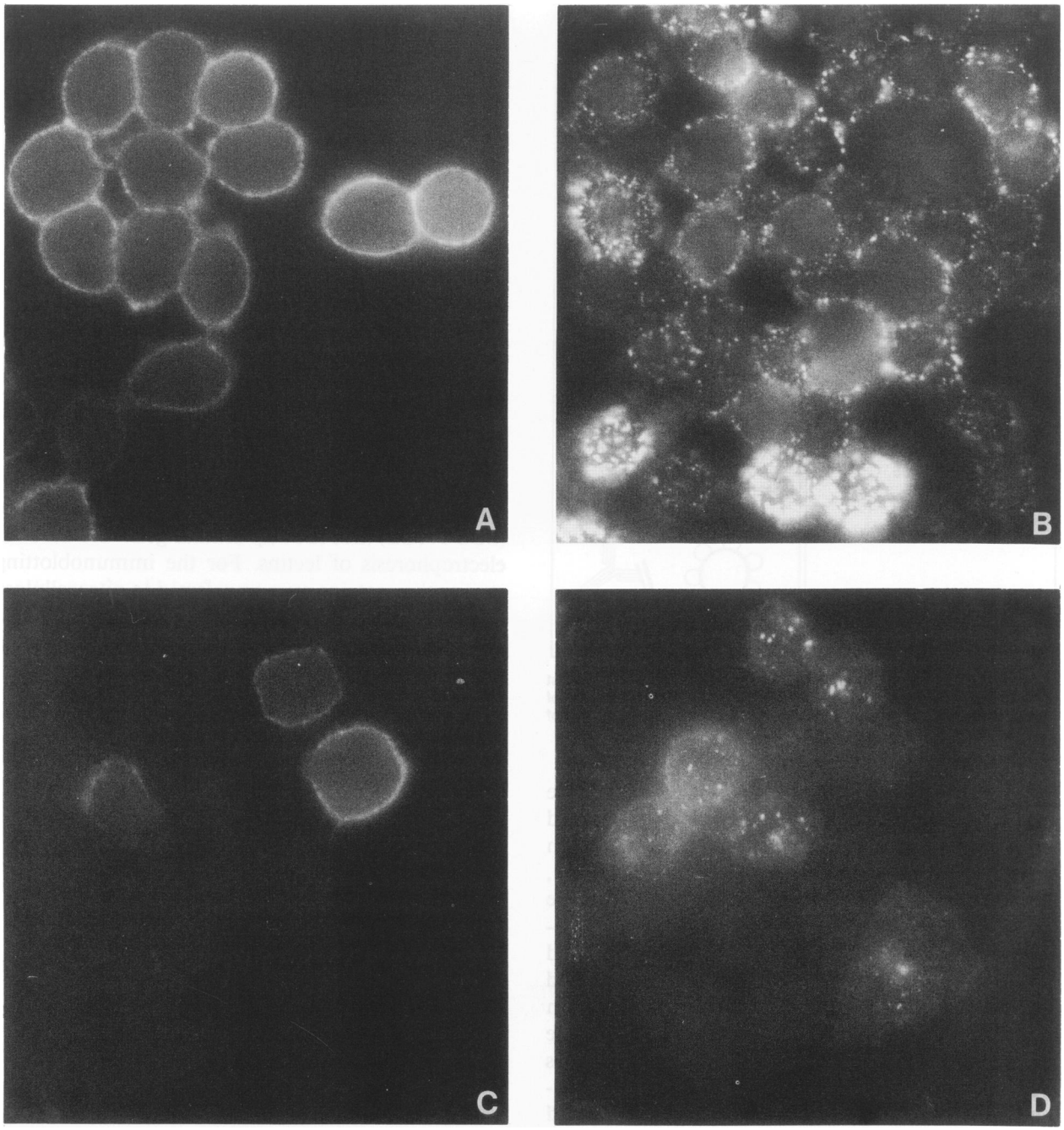

Figure 3—Immunostaining of HeLa cells. **A**—Cells were incubated with PNA followed by anti-PNA MAb (F3D12) and then by a fluoresceinated anti-mouse antibody. All the incubations were carried out on crushed ice. **B**—The protocol was the same but the temperature was 37 C. **C**—Cells incubated with fluoresceinated PNA only at 0 C. **D**—The same as **C** but at 37 C. (×600)

sclerosis (NS). The paraffin sections were deparaffinized through xylene and rehydrated through graded alcohols and PBS buffer (pH 7.6). Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 minutes in the course of rehydration, after absolute alcohol. Nonspecific staining was blocked with normal mouse serum (1:20) for 20 minutes after rehydration in PBS. Slides were incubated for 30 minutes with the MAb anti-Leu-M1 (1:10) from Beckton Dickinson. The slides stained with MAb F2C8 were prepared as follows: they were incubated for 30 minutes with highly purified PNA at a concentration of 0.02 mg/ml. A second incubation with MAb F2C8 (diluted 1:500, final concentration of purified IgG1, 48 μg/ml) was carried out for 30 minutes. Both sets of slides were incubated with biotinylated secondary antibody (1:

Table 2—Immunofluorescein Staining of Cell Lines Grown *in Vitro* With MAbs F2C8 or F2D12

Cell line	PNA Mabs	FITC-PNA
F9 (mouse teratocarcinoma)	+++	++
PYS (mouse parietal yolk sac cells)	+++	++
S4P-77 (small cell lung carcinoma)	+++	+
A549 (lung adenocarcinoma)	+++	+
MKN28 (gastric adenocarcinoma)	+	–
MKN45 (gastric adenocarcinoma)	++	+/–
NIH 3T3 (mouse fibroblast)	+	–
Schwann cells (mouse)	+	–
HeLa (human cervical carcinoma)	+++/–	+
HepG2 (human hepatoma)	++	–
Human fibroblast cells	+/–	–

These results refer to both the intensity of the staining and the number of cells that stained.

200) for 30 minutes and with avidin–biotin peroxidase complexes (Vectastain, ABC Kit, PK4002, Vector Laboratories) for 30 minutes; both incubations contained 3% normal serum. Each incubation was followed by three washings with PBS. Peroxidase reactivity was demonstrated with AEC (3-amino-9-ethyl-carbozole in N,N′-dimethylformamide) for 30 minutes. After two washings in PBS, the sections were counterstained with Mayer's hematoxylin for 1 minute and mounted in aqueous mounting medium. Negative controls were performed in each case by either replacing the primary antibody with normal nonimmune serum or omitting the avidin–biotin peroxidase complex.

Results

The protocol for screening hybridomas was aimed at selecting MAbs that would recognize that region of PNA not involved in binding to the sugar residues. We reasoned that the protocol illustrated in Figure 1 should yield two types of results. If the MAb under examination was not directed toward the active site of PNA, one would expect similar results whether the MAb was challenged with PNA or with PNA bound to Galβ1-3GalNAc-BSA. If, on the other hand, the MAb was directed toward the active site of PNA, we could expect that a stronger signal would be recorded when the lectin alone was used, instead of the lectin bound to the Galβ1-3GalNAc-BSA, because the MAb would be competing for the amino acid sequence interacting with the sugar.

All of the hybridomas screened fell into two classes, those that yielded stronger signals when the PNA was first bound to the Galβ1-3GalNAc-BSA, and those that gave a stronger signal when the MAbs were challenged with only PNA. We chose to characterize three clones: F2C8 and F3D12, which belonged to the first class of MAbs, and F3A5, which belonged to the second class of MAbs.

Biochemical Characterization of MAbs

All of the purified MAbs were tested for their ability to recognize PNA as well as other lectins in a dot blot and Western blot assay. All antibodies were able to recognize the native lectin, but F3A5 was also capable of recognizing denatured PNA (Figure 2). None of the antibodies recognized either native or denatured wheat germ agglutinin (WGA) (Figure 2). Native concanavalin A (Con A) and ricin were strongly stained only with MAb F3A5, but the denatured counterpart was moderately weakly or poorly stained, respectively (Figure 2). A summary of the dot tests studies is shown in Table 1. In these tests the concentration of the immunoglobulins was 7.5 μg/ml for F3A5 and 40 and 33 μg/ml for F3D12 and F2C8, respectively.

Studies were also carried out on the ability of the MAbs to recognize lectins after being transferred to nitrocellulose (Western blot analysis). In all cases it was impossible to detect the expected subunits of the lectins when the MAbs were used at the same concentration as that used for the dot assays. However, when the concentration of the MAbs was increased tenfold, all were able to recognize PNA, WGA, and both subunits of ricin (data not shown). Other lectins reacted only weakly or failed to react with the three MAbs, namely, Con A, soybean agglutinin, *Ulex europaeus* agglutinin, phytohaemoagglutinin, and pokeweed agglutinin (data not shown).

Using the competition assay described in the Materials and Methods section, we measured the affinity constants for the three MAbs for PNA: values of 3×10^8, 2.5×10^7, and 5×10^7 M^{-1} were calculated for F2C8, F3D12, and F3A5, respectively.

Staining Properties of MAbs

Similar results were obtained whether the cells were prefixed in acetone or fixed after the completion of the staining procedure. With F2C8 and F3D12, we stained 11 different cell lines with both MAbs. The fluorescence, when present, was always associated with the cell surface and was sensitive to the temperature at which the staining was carried out. At 0 C the staining was uniformly distributed, while at 37 C patches were observed (Figure 3A and B, respectively). When the same cells were stained with fluorescein-labeled PNA, the intensity of the staining was always reduced, although the pattern of staining at the two different temperatures was identical to that obtained on PNA-MAb-stained cells (Figure 3C and D).

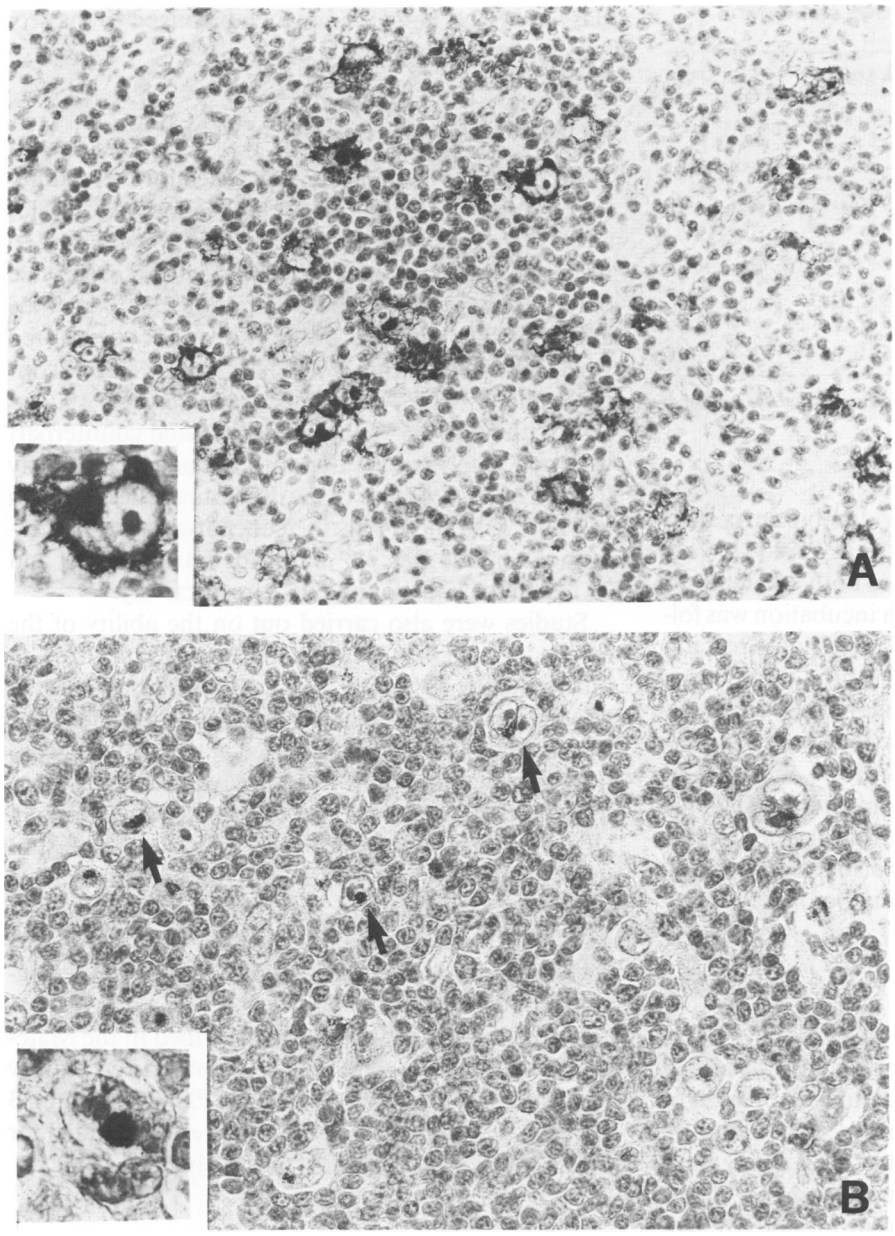

Figure 4—Immunostaining of RS cells in Hodgkin's disease. **A**—MAb F2C8 stains RS cells rather uniformly, and the stain is probably associated with the cell membrane. (ABC, ×235; **inset,** ×940) **B**—Anti-Leu-M1 staining is focused in the juxtanuclear region. (ABC, ×375; **Inset,** ×940) *Arrows* indicate some of the RS cells.

The increased sensitivity of the staining reaction for the different cell lines is shown in Table 2. Note that in several cases the cells failed to stain with FITC-PNA but strongly responded when stained with PNA MAbs. The staining with the MAb-PNA complex could be completely inhibited in the presence of free galactose, which indicated that the PNA was indeed binding to a galactosyl sugar moiety.

The staining properties of MAb F3A5 were anomalous, in that it was able to stain cells only after fixation and also in the absence of PNA, which suggested that this MAb recognized a prosthetic group common to proteins. The results of these studies will be presented elsewhere.

Comparison of MAb F2C8 With Anti-Leu-M1 in Lymph Nodes Derived From Patients With Hodgkin's Disease

Twenty-seven cases of Hodgkin's disease have been studied by immunohistochemical methods utilizing MAbs F2C8 and anti-Leu-M1 (Figure 4A and B, respectively). Using MAb F2C8, in all 27 cases at least some of the typical diagnostic cells, the RS cells were stained; and in most cases (19/27), more than 60% were positive (Table 3). In addition to these characteristic cells, the lymph nodes from Hodgkin's disease patients also contain variants, which we refer to as Sternberg-like cells. These cells usually contain lo-

Table 3—Comparison of PNA Binding Antigen and Leu-M1 Antigen in Hodgkin's Disease

	Reed-Sternberg cells		Sternberg-like cells	
Biopsy no.	Leu-M1	PNA	Leu-M1	PNA
LP				
80835	++	+++	0	+++
149730	0	+++	0	+++
205422	+++	+++	+++	+++
203900	+++	+++	+++	+++
222128	0	+++	0	+++
221048	+++	+++	+++	+++
225336	+	+++	+	+++
NS				
175559	+	+++	+	+
157587	++	+++	++	+++
64272	0	+	+	++
175588	0	+	+	++
69997	++	+++	+	+++
187973	+	++	+	+++
189467	++	+++	+	+++
212163	+	++	+	++
228639	+++	+++	++	+++
222270	0	+	0	+++
222269	+++	++	+++	++
220876	++	+++	++	+++
234513	+++	+++	+++	+++
244579	0	++	0	++
242640	+++	+++	+++	+++
211818	+++	+++	+++	+++
MC				
172634	0	+++	0	+++
194078	+++	+++	+++	+++
LD				
234512	+++	+	++	+
58563	0	++	++	++

Percent positive cells: 0, negative; +, <30%; ++, 30–60%; +++, >60%.

bated nuclei and small-to-medium-size nucleoli when compared with the RS cells.[43] These characteristics are illustrated in Figure 5. Immunohistochemical studies with MAb F2C8 were extended to the Sternberg-like cells. Essentially identical results were obtained and are summarized in Table 3. The results

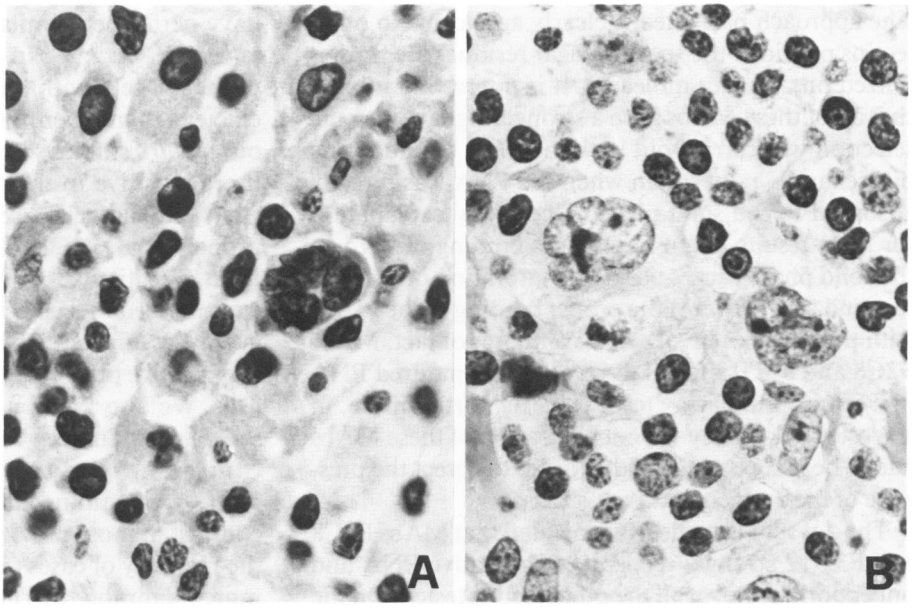

Figure 5—A classic RS cell (**A**) and Sternberg-like cells (**B**). (H & E, ×940)

Table 4—Staining Distribution of F2C8 and Leu-M1 Monoclonal Antibodies in Hodgkin's Disease Cells

	Positive cells*				Staining distribution†		
	Total	+	++	+++	F	D	F/D
Reed-Sternberg cells (n = 27)							
Leu-M1	19	4	5	10	16	1	2
PNA	27	3	4	20	1	26	—
Sternberg-like cells (n = 27)							
Leu-M1	21	7	5	8	17	3	—
PNA	27	3	7	17	22	5	—

* +, <30%; ++, 30–60%; +++, >60%.
† F, focused staining; D, diffused staining; F/D, both F and D.

with the anti-Leu-M1 antibody were very similar; however, with this antibody we did find several specimens that were negative: 8 of 27 for the RS cells and 6 of 27 for the Sternberg-like cells (Table 3).

During these studies we also observed that the distribution of the staining was remarkably different when the RS cells were compared with Sternberg-like cells. The MAb F2C8 stained the RS cells rather uniformly, and some of the stain was probably also associated with the cell membrane (Figure 4A and inset), while with the anti-Leu-M1 the staining was usually focused in the juxtanuclear region (Figure 4B and inset). This observation contrasts with the staining in Sternberg-like cells, which was mainly of the focused type whether anti-Leu-M1 or F2C8 was used. A summary of these results is illustrated in Table 4.

Discussion

In this paper we have presented a protocol for the production of MAbs against that region of PNA which is not involved with the binding to the sugar moiety. The approach presented is clearly applicable to other lectins provided the specific sugar residues are immobilized on a carrier molecule. It is interesting to note that all of these MAbs gave a stronger signal when the selection was carried out with the PNA bound to the disaccharide, rather than when the PNA was directly adsorbed to the solid phase. One likely explanation for these results might be that the binding of PNA to the solid phase causes partial denaturation of the protein and thus prevents the proper binding of the MAbs with the epitopes involved. As a matter of fact, MAbs F2C8 and F3D12 failed to recognize denatured PNA in a dot immunoassay test (Table 1); furthermore, in a Western blot assay the concentration of these MAbs had to be increased tenfold in order to detect the presence of the antigen (data not shown).

The dot spot tests clearly indicated that MAbs F2C8 and F3D12 strongly recognized the native PNA and only poorly or not at all the other lectins tested. On the other hand, MAb F3A5 recognized the native PNA, weakly the boiled protein, moderately strongly native Con A and ricin, but moderately weakly or poorly these two denatured lectins, respectively.

Our results with MAbs F2C8 and F3D12 showed that these antibodies have a considerable advantage over the PNA fluorescein method in the detection of the T antigen on the surface of cells grown *in vitro*. The data, summarized in Table 2, clearly indicate that using these MAbs, we were able to demonstrate the T antigen on the surface of all the cells tested, with the possible exception of human fibroblast cells. In these cells the reaction was of a dubious nature, being very weak and observable in only a few cells.

Studies on Hodgkin's Disease: Comparison of Anti-Leu-M1 and MAb F2C8

In the past several years the anti-Leu-M1 MAb, an anti-granulocyte antibody, has been used to identify the characteristic RS cells found in all Hodgkin's disease tissue.[20,27,29–31,39–41] A number of these workers have performed similar studies utilizing fluorescein-labeled PNA.[20,39,42] In all of these studies the staining of the RS cells with the anti-Leu-M1 MAb was more efficient than when the lectin was used.[20,39] Because our *in vitro* studies showed that our MAbs were much more sensitive in their ability to reveal surface antigen(s) in the cells used, we compared the staining properties of F2C8 with anti-Leu-M1 in lymph nodes from 27 patients with Hodgkin's disease. Our results, summarized in Table 3, showed that MAb F2C8 was at least as efficient in staining both RS cells as well as the putative precursor of these cells, the Sternberg-like cells. We also found a differential distribution of the staining within these cells (Table 4). Seven cases of the lymphocyte predominance Hodgkin's disease (LP-HD) were examined, and our results appear to be in contrast to those previously reported. In our hands, in the majority of cases (5 of 7) the rare RS cells and the more common Sternberg-like cells (4 of 7) were

stained with the anti-Leu-M1 MAb. On the other hand, other workers have reported that these diagnostic cells were generally not stained with this MAb.[20,27,28,30,31,40,41] Combined, these authors reported 41 cases of LP-HD; and in only 5 individuals were the diagnostic RS or the Sternberg-like cells stained with anti-Leu-M1. However, in one of these studies,[41] neuraminidase treatment permitted the staining of the Sternberg-like cells (the lymphocytic and histiocytic variants) with anti-Leu-M1 in 7 of 12 cases. Clearly the antigenic determinant of the anti-Leu-M1 MAb is present in the majority of these cells, but in a cryptic form. At the moment we cannot offer any reasonable explanation for finding, in the majority of our cases of LP-HD, the unmasked antigenic determinant of Leu-M1. It cannot, however, be excluded that the often subjective criteria used to classify the various types of Hodgkin's disease[43,44] may be partially responsible for the apparent discrepancy reported in the paper.

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