

Lymphoid Precursor Cells Adhere to Two Different Sites on Fibronectin

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Abstract. Several precursor lymphoid cell lines, blocked at specific stages of differentiation, adhere specifically to fibronectin in vitro. Whereas the Ba F3 cell line, which has both immunoglobulin heavy- and light-chain genes in germline configuration, interacts with the arg-gly-asp-containing cell-binding domain of fibronectin, the B-committed line PD 31, which is undergoing rearrangement of immunoglobulin light-chain genes, does not. Accordingly the Ba F3, but not the putative PD 31 surface fibronectin receptor, binds to an affinity matrix containing the 115-kD cell-binding domain of fibronectin. PD 31 cells recognize a different domain of the fibronectin molecule, which is contained within the carboxy terminal segment possessing

a high-affinity binding site for heparin. A polyclonal antibody raised against the fibronectin receptor of mouse erythroleukemic cells inhibits adhesion of these lymphoid lines to fibronectin. It precipitates two major species of 140 and 70 kD from surface-radiiodinated Ba F3 cells and species of 140 and 120 kD from PD 31 cells. We propose that the two types of cells express different fibronectin receptors mediating substrate adhesion, and suggest that receptor(s) with different specificity might be expressed in the course of B cell maturation. Because we show that these adhesion properties are shared by normal bone marrow lymphoid precursors, we infer that these receptors may play a role in normal lymphopoiesis.

WHEREAS the molecular mechanisms underlying immunoglobulin gene rearrangements are now understood at least in outline (Tonegawa, 1983) and the complex process of mature lymphocyte recirculation and homing is being actively investigated (Gowans and Knight, 1964; reviewed by Gallatin et al., 1986), remarkably little is known about the processes of membrane remodeling involved in the maturation of lymphoid precursors and in their release into the circulation. A link between successful rearrangement of antigen receptor genes and plasma membrane remodeling is likely, in that only a fraction of the cells produced daily in the maturative compartments survives and is released into the circulation (Gallatin et al., 1986).

In the normal adult mammal, hematolymphopoiesis is confined to the bone marrow, which provides a unique inductive microenvironment responsible for the ordered proliferation and differentiation of all the cell lineages whose mature progeny is found in the circulation (Zuckerman and Rhodes, 1985). Both stromal cells (Dexter et al., 1985) and the extracellular matrix (Zuckerman and Rhodes, 1985) play key roles in maintaining a balanced hemopoiesis, but the requirement for specific cellular and matrix factors is less understood.

Recent work has shown that both normal reticulocytes (Patel et al., 1985) and murine erythroleukemia cells (Patel and Lodish, 1984, 1986; Giancotti et al., 1986) possess a 140-kD fibronectin receptor recognizing the arg-gly-asp sequence within the cell-binding domain of fibronectin

(Pierschbacher et al., 1982; Yamada and Kennedy, 1984). Loss or modification of this receptor protein is responsible for the loss of adhesion to fibronectin observed in differentiated cells (Patel and Lodish, 1986). This receptor is similar in binding specificity to those described in human osteosarcoma cells (Pytela et al., 1985), mouse fibroblasts (Giancotti et al., 1985), platelets (Gardner and Hynes, 1985; Pytela et al., 1986) and chick embryo fibroblasts (Horwitz et al., 1985; Akiyama et al., 1986).

Here we show that several pre-B lymphoid cell lines grown in culture attach specifically to fibronectin in vitro, and provide evidence suggesting that this process is mediated by receptors that exhibit immunologic cross-reactivity with the erythroid receptor. At variance from uncommitted B cell precursors, which interact with the arg-gly-asp fibronectin recognition sequence, pre-B cells undergoing the rearrangement of heavy- and/or light-chain genes recognize a different region of the fibronectin molecule which is part of the carboxy-terminal end containing the high-affinity binding site for heparin. We suggest that fibronectin receptors with different specificity are expressed in the course of pre-B cell maturation. Because we also show that a significant fraction of normal bone marrow pre-B cells specifically adheres to fibronectin and to its carboxy-terminal fragment containing the high-affinity site for heparin but not the arg-gly-asp recognition sequence, we infer that multiple receptors may play a role in normal lymphoid precursor cell adhesion to the extracellular matrix.

Table I. Attachment of Lymphoid Cell Lines to Fibronectin In Vitro

Cell type	Ig genes		Surface Ig	Adherent cells	
	HC	LC		Fibronectin	BSA
				%	%
Ba F3	G	G	—	40.5	5.0
38 B9	pR	G	—	35.9	10.5
PD 31	R	pR	—	66.3	6.4
70 Z/3	R	R	—	75.4	31.0
WEHI 231	R	R	+	31.5	18.1
MPC 11	R	R	—	21.3	2.8
Circulating B lymphocytes*	R	R	+	<0.5	<0.5
MEL	—	—	—	83.9	5.3

Cells were assayed for attachment to fibronectin (5 µg/cm²) or BSA (2%) on 35-mm diameter plastic petri dishes. The entries under Ig genes and Surface Ig refer to the configuration of genes for immunoglobulin heavy chains (HC), light chains (LC), and to the presence (+) or absence (—) of surface Ig, respectively. Abbreviations: G, germline; pR, partially rearranged; R, rearranged. The cells are listed in order of their level of commitment along the B cell lineage. MEL cells were included as a positive control.

* Identified by immunofluorescence on a monocyte-depleted mononuclear fraction from peripheral blood.

Materials and Methods

Materials

Histopaque 1077, Nonidet-P40, cyanogen bromide-activated Sepharose 4B, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). Laminin was from Collaborative Research (Lexington, MA), Collagen type I from Flow Laboratories, Inc. (McLean, VA), *N*-acetyl-D-glucosamine from Pfanstiehl Laboratories (Waukegan, IL), wheat germ agglutinin-agarose from E. Y. Laboratories (San Mateo, CA), DEAE-Sepharose from Pharmacia Fine Chemicals (Piscataway, NJ), octyl-β-D-glucopyranoside from Calbiochem-Behring Corp. (San Diego, CA), and the synthetic peptides gly-arg-gly-asp-ser-pro-cys, gly-arg-gly-asp-ser-pro, and gly-arg-glu-asp-ser-pro from Peninsula Laboratories, Inc. (Belmont, CA). The rat RA3-6B2 monoclonal antibody was a generous gift of Dr. Robert Coffman (DNAX, Palo Alto, CA). Rabbit and goat IgG, goat affinity-purified IgG against mouse Ig, and F(ab)₂ fluorescein-conjugated goat anti-rat and rabbit anti-goat IgG were all from Cappel Laboratories (Malvern, PA); monoclonal antibodies against defined regions of fibronectin were purchased from Mallinckrodt Inc. (St. Louis, MO). Fibronectin was isolated from outdated human plasma by gelatin-Sepharose affinity chromatography according to Ruoslahti et al., (1982), and the 115-kD chymotryptic fragment containing the cell attachment domain was prepared according to Ruoslahti et al. (1981) and purified to >95% homogeneity as described (Patel and Lodish, 1986). A heparin-binding chymotryptic fragment of fibronectin was obtained by passing the chymotryptic digest in 0.15 M NaCl, 20 mM Tris, 1 mM CaCl₂ (pH 7.4) through a heparin-Sepharose column, and eluting the bound material with 1 M NaCl in the same buffer. A SDS-PAGE analysis of the eluted material is shown in the inset to Fig. 4 a.

Cell Cultures

Ba F3 cells, kindly provided by Dr. Bernard Mathey-Prevot of the Whitehead Institute, were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 10% of RPMI 1640 medium conditioned by the interleukin 3 (IL-3)-producing line WEHI 3. 38 B9 (Alt et al., 1981), PD 31 (Lewis et al., 1982), 70 Z/3 (Paige et al., 1978), and WEHI 231 (Ralph, 1979) cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS and 5.8 × 10⁻⁵ M 2-mercaptoethanol. MEL (Friend et al., 1971) and MPC 11 cells (Kuehl and Scharff, 1974) were grown in DME supplemented with 13% inactivated FCS. HepG2 and Chinese hamster ovary (CHO) cells were grown as monolayers in DME or Ham's F12, respectively, plus 10% heat-inactivated FCS, and detached with trypsin/EDTA (Gibco, Grand Island, NY). All growth media were supplemented with antibiotics. A brief description of the lymphoid lines used in this study is given in Table I.

Isolation of Bone Marrow Cells

Bone marrow cells were prepared from 4–5-w-old BALB/c mice from Dr.

R. Jaenisch's breeding colony at the Whitehead Institute. Femurs were isolated as described by Oliver and Goldstein (1978), and bone marrow cells were obtained by flushing the femurs with DME/5% FCS plus 5 mM Na-EDTA, and washed once in DME/5% FCS. A mononuclear fraction was prepared by standard Ficoll gradient centrifugation using Histopaque 1077. Cells at the DME-Ficoll interphase were collected, washed once in DME/5% FCS, and depleted of monocytes/macrophages by incubation for 1 h at 37°C in a tissue culture grade 60-mm diameter plastic dish (Falcon Labware, Oxnard, CA). Nonadherent cells were collected, and the dishes were flushed with a gentle stream of medium to detach loosely adherent cells which were pooled with the unattached fraction. This preparation contains 40–50% cells positive for the B lineage-restricted antigen B-220 (Coffman, 1982), as assayed by indirect immunofluorescence. Lymphocytes from mouse peripheral blood were isolated with the same procedure from 4-month-old mice of the same strain.

Attachment Assays

Adhesion assays of cells grown in suspension were carried out in either 35-mm diameter plastic petri dishes (Falcon Labware) or in 6-mm diameter, 96-well polystyrene plates (Linbro, Flow Laboratories, Inc.) exactly as described by Patel and Lodish (1984, 1986). Each experimental point is the average of triplicate (35-mm dishes) or quadruplicate determinations (96-well plates), and the standard deviation was within 10%. Adhesion assays of bone marrow cells and peripheral lymphocytes were carried out in 60-mm diameter petri dishes coated with the indicated amounts of fibronectin or its heparin-binding fragment, where 8 × 10⁶ cells per dish in 4 ml of DME/5% FCS were incubated at 37°C for 60 min. Nonadherent cells were collected and the dishes were rinsed twice with 2 ml of the same medium, which was pooled with the nonadherent cells. Adherent cells were detached by incubation in DME/5% FCS plus 5 mM EDTA at 4°C for 20 min. Both adherent and nonadherent cells were counted with a Coulter counter (Coulter Electronics Inc., Hialeah, FL), and further analyzed by indirect immunofluorescence.

Immunofluorescence

Cells were washed once in Hank's balanced salt solution supplemented with 20 mM Hepes and 1% bovine serum albumin (BSA), resuspended in 1 ml of the same solution, and reacted for 15 min on ice with (a) 1 mg/ml control IgG of the same species as that of the secondary fluorescent antibody, (b) RA3-6B2 rat monoclonal antibody (Coffman, 1982) or affinity-purified IgG fraction of goat anti-mouse Ig, and (c) fluoresceinated F(ab)₂ fragments of either goat anti-rat or rabbit anti-goat IgG. Cells were washed with Hank's balanced salt solution/20 mM Hepes/1% BSA after each step, and finally resuspended in 0.15 M NaCl, 10 mM Tris-HCl, 0.2% NaN₃ (pH 8.0). Fluorescent cells were counted with a Zeiss III RS epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). At least 200 cells were counted, and

unspecifically stained cells were <0.5%, as determined on controls not treated with the primary antibody.

Surface Iodination and Immunoprecipitation

Cells were surface-labeled with ^{125}I -sodium iodide (100 mCi/ml, Amersham Corp., Arlington Heights, IL) according to Sefton et al. (1973), and extracted with 0.15 M NaCl, 10 mM Na-phosphate, 1 mM CaCl_2 , 1 mM MgCl_2 , 2 mM PMSF, 1 mM NaI, 0.2 M octylglucoside (pH 7.4) as described by Patel and Lodish (1986). In this study, we used 1 mCi of ^{125}I and 1 ml of extraction buffer per 5×10^7 cells with the exception of the experiment reported in Fig. 5, where 3 mCi of ^{125}I was used. For immunoprecipitation, 0.5 ml of total cell extract or affinity-purified fibronectin receptor was added to 1 ml of 0.15 M NaCl, 20 mM Tris-HCl pH 7.5, 1 mM CaCl_2 , 2 mM PMSF, 2 mg/ml BSA, 1% NP 40. Then, 30 μl of preimmune or antifibronectin receptor antiserum was added, and the samples were incubated for 90 min at 4°C on a rocking platform. Then 50 μl per sample of 10% *Staphylococcus aureus* protein A was added, and the incubation was continued for a further 30 min. The samples were centrifuged for 20 s at 13,000 g, and the precipitates were washed sequentially with the same buffer, then with 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM CaCl_2 , 1 mM PMSF, 0.1% NP 40, and finally with 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5. The washed precipitates were dissolved in Laemmli (1970) gel sample buffer, boiled for 3 min, and centrifuged at 13,000 g for 1 min at room temperature. The supernatants were subjected to SDS-PAGE as specified in the figure legends. Mouse erythrocyte membranes labeled with ^{125}I in the presence of 0.05% Triton X-100 and/or myosin, β -galactosidase, phosphorylase B, BSA, ovalbumin, and carbonic anhydrase were used as molecular mass markers. The gels were dried and autoradiographed at -70°C with an intensifying screen with either Kodak XAR or SB5 films (Eastman Kodak Co., Rochester, NY), as specified in the figure legends.

Purification of the Fibronectin Receptor

An octylglucoside cell extract (2 ml) from ^{125}I surface-labeled MEL cells (10^8 cell equivalents) was chromatographed on 1-ml bed volume of wheat germ agglutinin-agarose equilibrated with 50 mM NaCl, 10 mM Na-phosphate, 1 mM CaCl_2 , 1 mM MgCl_2 , 1 mM PMSF, and 50 mM octylglucoside (pH 7.4). After extensive washing of the column, bound material was eluted with 0.5 M *N*-acetylglucosamine in the same buffer, and loaded onto 1-ml bed volume of DEAE-sepharose preequilibrated with 10 mM Na-phosphate, 1 mM CaCl_2 , 1 mM MgCl_2 , 1 mM PMSF, and 50 mM octylglucoside (pH 7.4). After washing the column with the same buffer, bound material was eluted with 0.17 M NaCl, 10 mM Na-phosphate, 1 mM CaCl_2 , 1 mM MgCl_2 , 1 mM PMSF, 50 mM octylglucoside (pH 7.4) and the eluate was loaded on an affinity matrix (1-ml bed volume) obtained by coupling the 115-kD chymotryptic fragment of fibronectin to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions (Pharmacia technical handbook, 1985). The column was washed with 0.15 M NaCl, 10 mM Na-phosphate, 1 mM CaCl_2 , 1 mM MgCl_2 , 1 mM PMSF, 50 mM octylglucoside (pH 7.4), and the fibronectin receptor was eluted with a 1 mg/ml solution of the heptapeptide gly-arg-gly-asp-ser-pro-cys dissolved in the same buffer. The eluted material, which migrated as a broad band of ~ 140 kD on reducing gels, was concentrated by adsorption on wheat germ agglutinin-agarose and eluted with 1 ml of 0.5 M *N*-acetylglucosamine. A 30- μl aliquot of the eluate was mixed with an equal volume of double-strength Laemmli gel sample buffer and boiled for 3 min, while the remainder was immunoprecipitated with antifibronectin receptor antibody (see Fig. 7). The antiserum was obtained by immunizing rabbits with the material eluted from the 115-kD Sepharose column after following the same protocol described above with unlabeled cells.

For the experiment reported in Fig. 5, an octylglucoside cell extract (1 ml each) from ^{125}I surface-labeled Ba F3 and PD 31 cells (5×10^7 cell equivalents) was chromatographed directly on 1-ml bed volume columns containing the 115-kD fragment of fibronectin coupled to a Sepharose matrix. After washing with 7 ml of 0.15 M NaCl, 10 mM Na-phosphate, 1 mM CaCl_2 , 1 mM MgCl_2 , 1 mM PMSF, 50 mM octylglucoside (pH 7.4), the columns were sequentially eluted with 2 ml of a 1 mg/ml solution of gly-arg-gly-glu-ser-pro peptide, 2.5 ml of a 1 mg/ml solution of gly-arg-gly-asp-ser-pro peptide, and finally 2 ml of 6 M urea, all in the above buffer. Fractions of 0.5 ml were collected, and 40 μl of each was mixed with an equal volume of double-strength Laemmli gel sample buffer, boiled for 3 min, and subjected to SDS-PAGE and autoradiography as specified in the legend to Fig. 5.

Results

Attachment of Selected Lymphoid Lines to Fibronectin In Vitro

Six cell lines of B lymphoid lineage blocked at specific stages of differentiation were screened for their ability to attach to fibronectin-coated dishes (Table I). Ba F3 is an interleukin 3-dependent clone that is positive for the B lineage-restricted B220 antigen, and has the immunoglobulin genes in germline configuration. Clones with similar properties have been shown to undergo in vivo differentiation along the B lineage (Palacios and Steinmetz, 1985), and therefore Ba F3 likely represents a B stem cell. 38 B9 is an Abelson transformant derived from fetal mouse liver that has undergone heavy- but not light-chain gene rearrangement (Alt et al., 1981). PD 31 is an Abelson-transformed cell line derived from adult mouse bone marrow that in vitro actively undergoes light-chain gene rearrangement (Lewis et al., 1982). 70 Z/3 is a chemically transformed pre-B cell line that can be induced to express surface immunoglobulin in vitro (Paige et al., 1978), whereas WEHI 231 is a lymphoma line constitutively expressing high levels of surface IgM (Ralph, 1979). MPC 11 is an IgG-producing mouse myeloma line (Kuehl and Scharff, 1974). MEL is a Friend virus-transformed erythroleukemic line (Friend et al., 1971), which was used here as a positive control, in that it expresses a well-characterized receptor for fibronectin (Patel and Lodish, 1984, 1986).

The rationale for using these different lymphoid cell lines was to determine whether adhesion to fibronectin is developmentally regulated. Data in Table I and Fig. 1 suggest that adhesion to fibronectin decreases with differentiation. A greater extent of adhesion (40–70%) was observed with the pre-B lines, whereas the WEHI 231 and MPC 11 cells yielded the lowest values (20–30%) and B lymphocytes from peripheral blood did not adhere at all. Note that 70 Z/3 and WEHI 231 cells exhibited a relatively high attachment to BSA-coated dishes, which contributes to an overestimation of the degree of adhesion to fibronectin inasmuch as albumin is used to block unspecific protein adsorption sites after coating the dishes with fibronectin. Attachment to fibronectin is specific, because neither laminin nor type I collagen promoted comparable levels of adhesion (not shown). These findings suggest that the specific attachment to fibronectin is

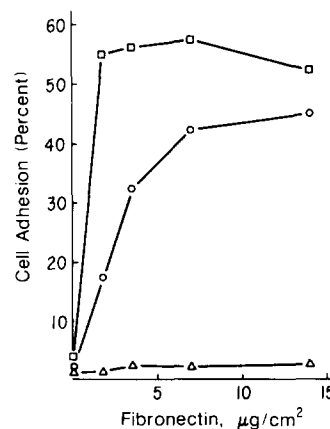


Figure 1. Dose-dependent attachment of selected cell lines to fibronectin. 96-well polystyrene microtiter plates (Linbro) were coated with the indicated amounts of fibronectin, and a cell attachment assay carried out with MEL (squares), PD 31 (circles), or MPC 11 (triangles) cells.

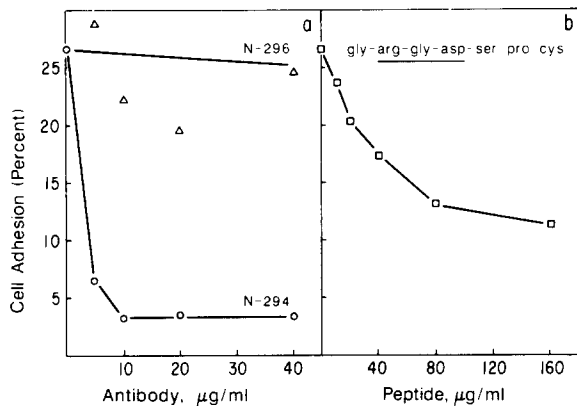


Figure 2. Effect of antifibronectin antibodies and of a synthetic peptide on Ba F3 cell attachment to fibronectin. 96-well polystyrene plates were coated with 10 µg/cm² fibronectin. (a) N-296 (triangles) or N-294 (circles) monoclonal antibodies were added, and the plate incubated for 1 h at 4°C before addition of Ba F3 cells. (b) The indicated concentrations of the synthetic peptide gly-arg-gly-asp-ser-pro-cys were added immediately before the addition of cells.

higher in pre-B cells than in surface Ig-positive or Ig-producing cells.

The difference between the two groups was more striking if the assay was carried out in 96-well microtiter plates rather than in 35-mm diameter petri dishes. Fig. 1 shows that the pre-B line PD 31 exhibited a dose-dependent attachment to fibronectin, whereas the myeloma line MPC 11 did not. A likely explanation is that the geometry of the vessel, and hence the hydrodynamic forces to which the cells are subjected during the washes, can cause decreased recovery of the adherent fraction.

The fibronectin receptor of most cell types characterized thus far specifically recognizes and interacts with the arg-gly-asp fibronectin sequence contained in the cell-binding domain of the protein (reviewed by Ruoslahti and Pierschbacher, 1986). Fig. 2 shows that cells of the lymphoblastoid Ba F3 clone bind to the same domain of fibronectin. Fig. 2 a shows that the monoclonal antibody N-294, directed against a pepsin cleavage site at the amino terminus of the 11.5-kD cell adhesion fragment of fibronectin, completely inhibited Ba F3 attachment, whereas the N-296 antibody, which is directed against the carboxy terminus of fibronectin which contains the second fibrinogen-binding site, did not interfere even at 80 µg/ml (not shown). Similar results were obtained

with MEL cells (Patel and Lodish, 1986). In keeping with these findings, the synthetic heptapeptide gly-arg-gly-asp-ser-pro-cys inhibited cell attachment to fibronectin in a dose-dependent fashion (Fig. 2 b) whereas a control gly-arg-glu-asp-ser-pro peptide was without effect (not shown).

Lack of Adhesion of Committed Lymphoid Precursor Cell Lines to the Arg-Gly-Asp Signal, but Adhesion to the Carboxy-terminal Region of Fibronectin Containing the High-affinity Heparin-binding Domain

To further characterize the interaction of selected lymphoid lines with fibronectin, we tested the ability of Ba F3 and PD 31 cells to adhere to a 115-kD chymotryptic fragment of fibronectin containing the arg-gly-asp cell attachment recognition sequence (Ruoslahti and Pierschbacher, 1986).

Fig. 3 a shows that Ba F3 cells attached equally well to intact fibronectin and to its 115-kD chymotryptic fragment, much like MEL cells, used here as a positive control (Fig. 3 c). Interestingly, PD 31 cells attached to intact fibronectin but not to the 115-kD fragment (Fig. 3 b), suggesting that either these cells do not recognize the arg-gly-asp sequence or that they require additional site(s) for effective adhesion. This finding was confirmed unequivocally in assays in 35-mm dishes, and in experiments where [¹⁴C]leucine-labeled PD 31 cells were plated together with [³H]leucine-labeled MEL cells (not shown). Results similar to PD 31 cells, not shown here, were obtained with the early pre-B line 38 B9, suggesting that this might be a more general feature of committed B cells.

To identify the region of the fibronectin molecule involved in the adhesion of PD 31 cells, we analyzed fragments from different regions of the fibronectin molecule for adhesion-promoting activity. Dose-dependent attachment of PD 31 cells was promoted by a preparation purified by heparin-Sepharose affinity chromatography of a chymotryptic digest of fibronectin (Fig. 4 a). A SDS-PAGE analysis of this preparation is shown in the inset to Fig. 4 a. Under reducing conditions (lane 1), four major polypeptides of 43, 32, 29, and 17 kD were detected. Under nonreducing conditions (lane 2), the 43- and 17-kD species migrated as a single band of 55 kD, whereas the 29–32-kD doublet mobility was unaffected. Western blots of this preparation, not shown here, revealed that none of these fragments is recognized by the N-294 monoclonal antibody. The 43-kD species is recognized by antibody N-296 and therefore encompasses the carboxy-terminal end of fibronectin containing the second fibrin-binding site. Therefore we infer that the 29–32-kD

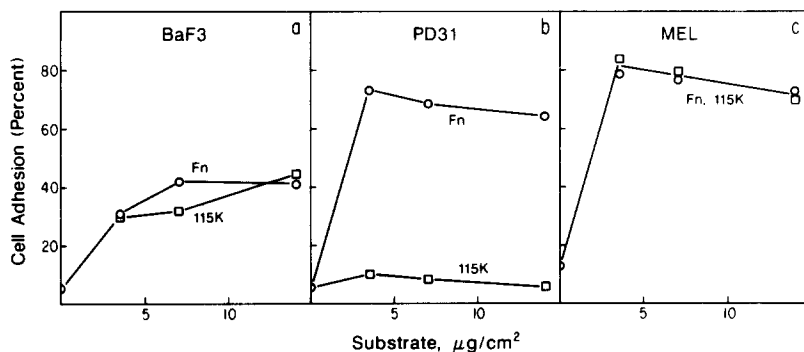


Figure 3. Adherence of selected cell lines to intact fibronectin or to its 115-kD chymotryptic fragment. 96-well polystyrene plates (Linbro) were coated with the indicated amounts of fibronectin (circles) or its 115-kD chymotryptic fragment (squares), and a cell-binding assay was carried out with (a) Ba F3, (b) PD 31, or (c) MEL cells.

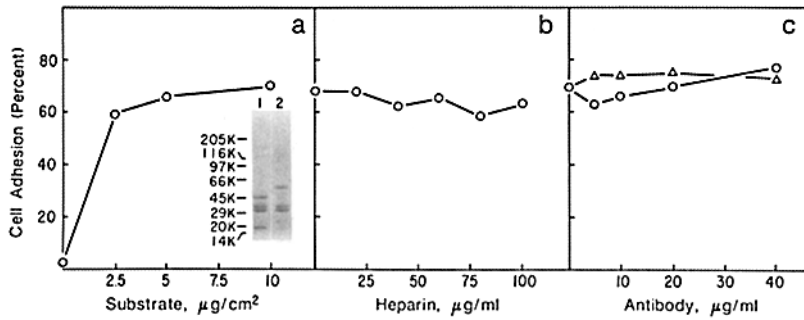


Figure 4. Adherence of PD 31 cells to a heparin-binding fragment of fibronectin. Lack of inhibition by heparin and by antifibronectin antibodies. 96-well polystyrene plates were coated with (a) the indicated amounts or (b and c) with $4 \mu\text{g}/\text{cm}^2$ of a heparin-binding chymotryptic fragment of fibronectin. A cell-binding assay was carried out (a) with PD 31 cells without further additions or in the presence of (b) the indicated concentrations of heparin or (c) antifibronectin monoclonal antibody N-296 (triangles) or N-294 (circles). The inset to panel a shows a Coomassie Blue-stained SDS-PAGE analysis of the heparin-binding preparation on a 5–15% gradient gel under (lane 1) reducing or (lane 2) nonreducing conditions. K, M_r standards in kilodaltons.

doublet is part of the high-affinity heparin-binding site of fibronectin, and is located between the carboxy-terminal fibrin-binding site and the cell-binding site.

Fig. 4 also shows that adhesion of PD 31 cells to microtiter wells coated with this heparin-binding preparation was not inhibited by either heparin (b) or by the N-294 or N-296 antifibronectin antibodies (c). Thus, it seems unlikely that adhesion is mediated by surface heparan proteoglycans or by trace contaminants that derive from the classical “arg-gly-

asp” cell-binding domain. Rather, these experiments suggest that PD 31 cells express a fibronectin receptor with a different specificity.

To test this hypothesis at the molecular level, we subjected extracts from surface-radioiodinated Ba F3 and PD 31 cells to affinity chromatography on a matrix obtained by coupling the 115-kD chymotryptic fibronectin fragment to Sepharose (Pytela et al., 1985). Fig. 5 shows that the synthetic gly-arg-gly-asp-ser-pro peptide eluted a band of ~ 140 kD from Ba

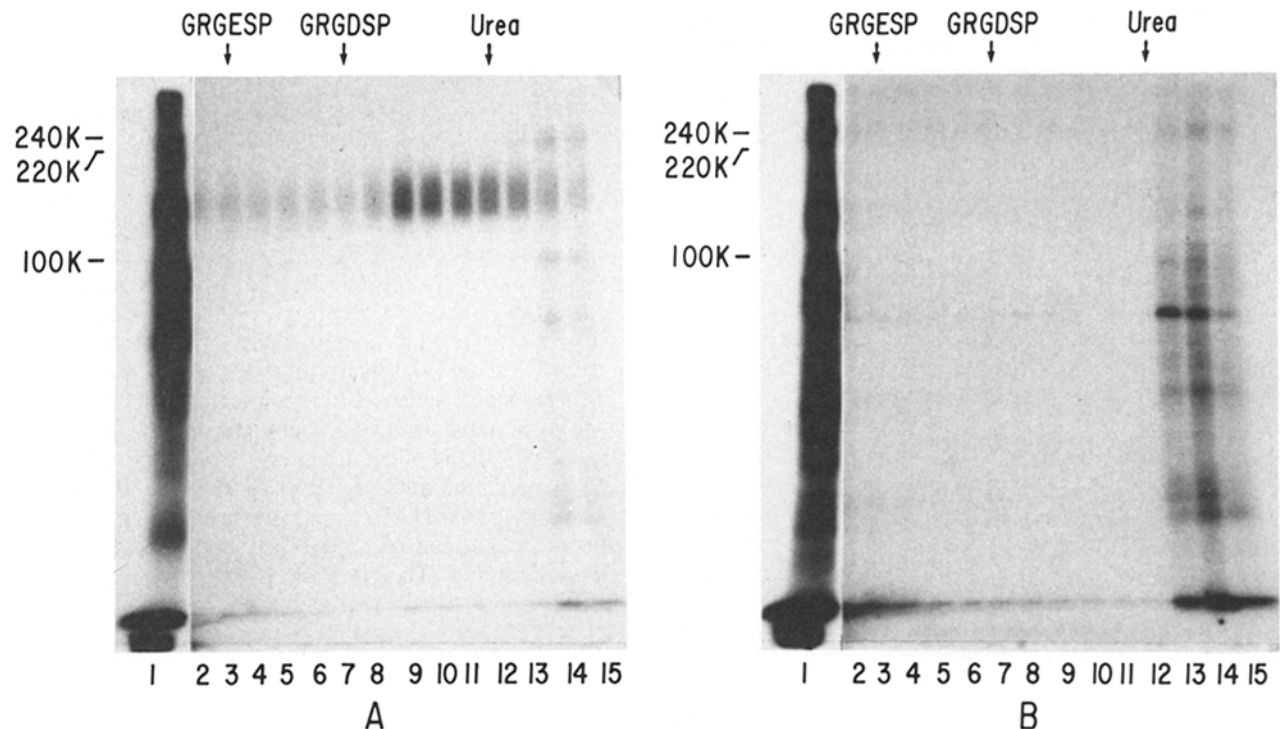


Figure 5. Autoradiograms of SDS-PAGE analyses of fractions obtained by affinity chromatography of cell extracts from surface radioiodinated cells on the 115-kD chymotryptic fragment of fibronectin. Extracts from ^{125}I surface-labeled Ba F3 and PD 31 cells (1 ml) were prepared, and chromatographed on 1-ml bed volume columns obtained by coupling a 115-kD chymotryptic fragment of fibronectin to Sepharose 4B. After washing, the columns were sequentially eluted (arrows) with 2 ml of a 1 mg/ml solution of gly-arg-gly-glu-ser-pro peptide (GRGESP), 2.5 ml of a 1 mg/ml solution of gly-arg-gly-asp-ser-pro (GRGDSP) peptide, and 2 ml of 6 M urea. Fractions of 0.5 ml were collected, and $40 \mu\text{l}$ of each analyzed on 7.5% Laemmli gels under reducing conditions. (A) Ba F3 cells; (B) PD 31 cells. In each panel: lane 1, flowthrough; lane 2, last wash fraction before elution; lanes 3–6, elution with GRGESP peptide; lanes 7–11, elution with GRGDSP peptide; lanes 12–15, elution with urea. The dried gels were exposed to Kodak XAR-5 film at -70°C with an intensifying screen. Exposure time was 8 h for lane 1 (in each panel) and 4 d for lanes 2–15. Specific activity of the extracts was (trichloroacetic acid-precipitable counts $\times \mu\text{l}^{-1}$): Ba F3, 94,400; PD 31, 85,100. K, M_r standards in kilodaltons.

F3 cells (A) but not from PD 31 cells (B). This finding is in good agreement with the cell adhesion experiments, and strongly suggests that the putative fibronectin receptor of the committed lymphoid line PD 31 does not recognize the argly-asp cell recognition sequence on fibronectin.

Characterization of an Antiserum against the Fibronectin Receptor of Mouse Erythroleukemia Cells: Immunologic Cross-reactivity with the Putative Lymphoid Receptor

To further characterize the fibronectin receptor(s) of mouse erythroleukemia cells (Patel and Lodish, 1984, 1986) and, we hoped, of B cells, an antiserum against the affinity-purified erythroid fibronectin receptor was raised in rabbits. Fig. 6 shows that a purified Ig fraction of this antiserum inhibited attachment of MEL cells (a), Ba F3 cells (b), and PD 31 cells (c) to fibronectin, whereas preimmune Ig were without effect. Even though in the case of PD 31 cells the degree of inhibition was lower and a higher concentration of antibody was required, these results were reproducible.

To identify cell surface components recognized by this antiserum, a detergent extract of ¹²⁵I surface-labeled MEL cells was prepared; part was immunoprecipitated with either preimmune or with antifibronectin receptor serum, while the remainder was subjected to an affinity chromatography purification procedure as detailed in Materials and Methods (Patel and Lodish, 1986). As shown in Fig. 7, the specific immunoprecipitates from the total cell extract (lane 3) and the affinity-purified receptor (lane 5) contained the 140-kD protein characterized previously as the erythroid fibronectin receptor (lane 4) (Patel and Lodish, 1986). Note that the immunoprecipitate from the total cell extract (lane 3) appears to migrate slightly faster than the affinity-purified receptor (lanes 4 and 5). This might be due to limited proteolysis of the labeled protein during incubation of the total cell extract with antibody. Alternatively, the radioactive iodine might have labeled a lower molecular mass precursor form of the receptor in a small number of permeable cells. Since the

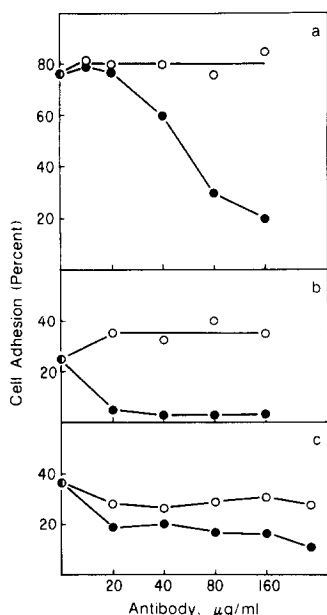


Figure 6. Inhibition of cell attachment to fibronectin by antibodies against the fibronectin receptor of MEL cells. 96-well polystyrene plates were coated with (a) 5 µg/cm² or (b and c) 10 µg/cm² of fibronectin. (a) MEL, (b) Ba F3, or (c) PD 31 cells were preincubated for 1 h at 0°C with the indicated concentrations of a purified Ig fraction from rabbit sera obtained before (open circles) or after (solid circles) immunization with affinity-purified fibronectin receptor from MEL cells.

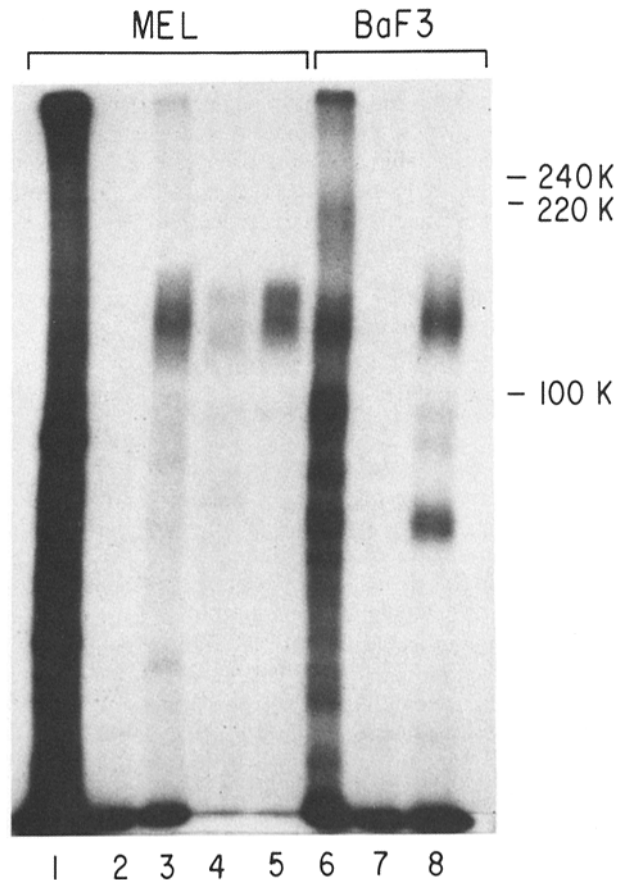


Figure 7. Autoradiogram of a SDS-PAGE analysis of immunoprecipitates obtained from total cell extracts or from affinity-purified fibronectin receptor with an antiserum against the MEL fibronectin receptor. Extracts from ¹²⁵I surface-labeled MEL and Ba F3 cells were prepared, and the same number of cell equivalents was immunoprecipitated with rabbit serum obtained before or after immunization with purified fibronectin receptor from MEL cells. Part of the same MEL extract was used to affinity-purify the fibronectin receptor, which was then immunoprecipitated with immune serum. Samples were analyzed by SDS-PAGE on a 7.5% polyacrylamide gel under reducing conditions. Lanes 1-5, MEL cells: lane 1, total cell extract; lane 2, immunoprecipitate of total cell extract with preimmune serum; lane 3, immunoprecipitate of total cell extract with immune serum; lane 4, affinity-purified fibronectin receptor; lane 5, immunoprecipitate of affinity-purified fibronectin receptor with immune serum. Lanes 6-8, Ba F3 cells: lane 6, total cell extract; lane 7, immunoprecipitate of total cell extract with preimmune serum; lane 8, immunoprecipitate of total cell extract with immune serum. The dried gel was exposed to Kodak XAR film for 5 d at -70°C with an intensifying screen. K, M, standards in kilodaltons.

precursor does not bind to the 115-kD Sepharose affinity matrix but is recognized by this antibody (V. P. Patel, unpublished), the immunoprecipitate from the total cell extract could be expected to appear broader. A protein of ~140 kD was also immunoprecipitated with immune serum from a detergent extract from ¹²⁵I surface-labeled Ba F3 cells (lane 8) and from affinity-purified Ba F3 fibronectin receptor (not shown), suggesting that the adhesion of Ba F3 cells to fibronectin was mediated by a similar receptor. In addition to the 140-kD protein, a second major protein of 70 kD was

immunoprecipitated from the Ba F3 cell extract. This protein was not present in the RGD eluate from the fibronectin affinity column (Fig. 5 a, lanes 7-11). Whether these proteins are functionally related, or whether the lower molecular mass form is a proteolytic product is unclear at present. In any case, the availability of the antifibronectin receptor antiserum allowed us to screen a large number of lymphoid lines for the presence of related surface molecules.

Immunoprecipitation of ^{125}I Surface-labeled Lymphoid Cell Extracts with Antifibronectin Receptor Serum

To test whether cells of B lineage express proteins immunologically related to the erythroid fibronectin receptor, we im-

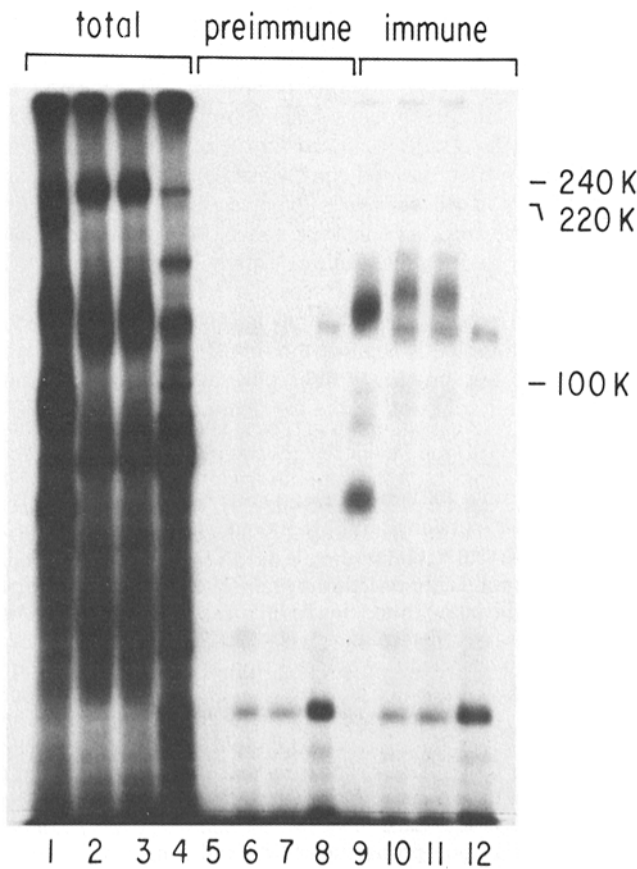


Figure 8. Autoradiogram of a SDS-PAGE analysis of immunoprecipitates from selected lymphoid lines with antifibronectin receptor antiserum. Extracts from 5×10^7 ^{125}I surface-labeled cells were prepared, and identical aliquots (0.5 ml) immunoprecipitated with either preimmune or immune serum against the MEL fibronectin receptor. The samples were analyzed by SDS-PAGE on a 7.5% polyacrylamide gel under reducing conditions. Lanes 1-4, total cell extracts (2.5×10^5 cell equivalents); lanes 5-8, immunoprecipitates with preimmune serum (from 2.5×10^7 cell equivalents); lanes 9-12, immunoprecipitates with immune serum (from 2.5×10^7 cell equivalents). Lanes 1, 5, and 9, Ba F3 cells; lanes 2, 6, and 10, 38 B9 cells; lanes 3, 7, and 11, PD 31 cells; lanes 4, 8, and 12, MPC 11 cells. The dried gel was exposed to Kodak SB5 film for 52 h at -70°C with an intensifying screen. Specific activity of the extracts was (trichloroacetic acid-precipitable counts $\times \mu\text{l}^{-1}$): Ba F3, 78,400; 38 B9, 41,700; PD 31, 54,600; MPC 11, 73,600. K, M_r standards in kilodaltons.

munoprecipitated with the antifibronectin receptor antiserum extracts from selected, ^{125}I surface-labeled cell lines. Fig. 8 illustrates the results we obtained with Ba F3, 38 B9, and PD 31, which adhere to fibronectin, and MPC 11, which does not. Ba F3 cells (lane 9) showed two major bands similar to those described in Fig. 7, lane 8, whereas 38 B9 and PD 31 cells exhibited two major bands of 140 and 120 kD, and a minor component of higher molecular mass (lanes 10 and 11). None of these proteins was present in the precipitates with preimmune serum (lanes 5-8). On the other hand, the immune serum precipitated no species from MPC 11 cells (lane 12) that were not precipitated by preimmune serum (lane 8). In the experiment described in Fig. 8, the high molecular mass species of Ba F3 cells showed a faster mobility on the SDS-PAGE gel than did the 140-kD species from 38 B9 and PD 31 cells. This may be due to differences in protein glycosylation, since digestion of Ba F3 and PD 31 immunoprecipitates with *N*-glycanase (but not *O*-glycanase) produced a shift of the 140- and 120-kD proteins to a single band of ~ 100 kD (not shown). The absence of reactive material in MPC 11 cells was not due to differences in the specific activity of the radioiodinated extracts, because in this particular experiment the specific activity of the Ba F3 and MPC 11 extracts was the same, and about twice that of 38 B9 and PD 31 cells (compare also lanes 1-4). Thus, this myeloma line did not attach to fibronectin and did not express proteins specifically recognized by the antiserum against the erythroid fibronectin receptor. On the other hand, the Ba F3, 38 B9, and PD 31 lines, which adhered to fibronectin, expressed species that are immunologically cross-reactive with the erythroid receptor molecule.

Fig. 9 compares the electrophoretic pattern of immunoprecipitates from ^{125}I surface-labeled Ba F3, 38 B9, and PD 31 cells under reducing and nonreducing conditions in separate 9% polyacrylamide gels. Under reducing conditions, the Ba F3 cells exhibited the 140- and 70-kD proteins (lane 4), and 38 B9 and PD 31 displayed three bands of 180, 140, and 120 kD (lanes 5 and 6, a-c). The 140- and 120-kD species (b and c) were always detected in repeats of this experiment, while the 180-kD species (a) was not, and its origin is unclear at present. Under nonreducing conditions there was a definite difference in mobility: a major, broad band (arrow) was detected in 38 B9 and PD 31 immunoprecipitates (lanes 2 and 3), which migrated near the higher molecular mass species from Ba F3 cells (lane 1). These results were confirmed in a similar experiment where the immunoprecipitates were analysed on the same 7.5% polyacrylamide gel (not shown).

Normal Bone Marrow Pre-B Cells Adhere to Fibronectin and to Its Heparin-binding Fragment *In Vitro*

Expression of cell surface receptors mediating adhesion to fibronectin is a remarkable feature of these transformed B cell lines that are adapted to growth in suspension culture. To determine whether this property is related to the transformed phenotype, or whether these cells were constitutively expressing the receptor molecules when the transformation event(s) occurred, we have extended our analysis to cells from normal bone marrow, which is the site of maturation of lymphocytes in the adult mammal (Osmond and Nossal, 1974; Osmond, 1975). We prepared a fraction enriched in mononuclear cells by density gradient centrifugation, and

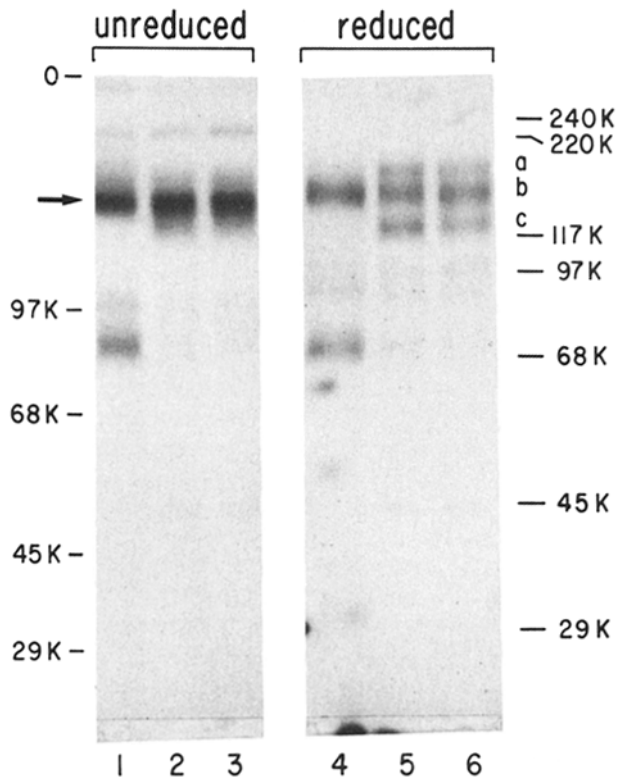


Figure 9. Autoradiogram of a SDS-PAGE analysis of immunoprecipitates from selected lymphoid lines with antifibronectin receptor serum. Comparison between reducing and nonreducing conditions. Extracts from ^{125}I surface-labeled cells were immunoprecipitated with antifibronectin receptor serum. The immunoprecipitates were dissolved in gel sample buffer and divided into two identical aliquots, one of which was reduced with 2-mercaptoethanol. The samples were boiled for 3 min and subjected to SDS-PAGE on separate 9.0% polyacrylamide gels. Lanes 1-3, unreduced samples; lanes 4-6, reduced samples. Lanes 1 and 4, Ba F3 cells; lanes 2 and 5, 38 B9 cells; lanes 3 and 6, PD 31 cells. The dried gels were exposed to Kodak XAR film for 23 h at -70°C with an intensifying screen. o, origin of gels. K, M_r standards in kilodaltons.

this fraction was depleted of monocytes and macrophages by adherence to tissue culture grade plastic dishes. This preparation is enriched in cells of B lineage, in that 40–50% of the cells were positive for the antigen B220, which is a B-restricted antigen expressed throughout the life cycle of these cells (Coffman, 1982). Table II shows that 55% of the B220-positive cells and 26% of the surface Ig-positive cells adhered to intact fibronectin, whereas 94% of the B220-positive cells and 92% of the surface Ig-positive cells adhered to the heparin-binding fragment of fibronectin. Why a population of bone marrow cells adheres to the heparin-binding fragment but not to intact fibronectin is unclear at present, but even with PD 31 cells we have routinely observed that a larger number of cells adhere to the heparin-binding fragment. Attachment of bone marrow pre-B cells to fibronectin was specific, in that they did not adhere to either laminin or collagen type I, nor did they show an increased attachment on a combination of fibronectin and collagen (not shown). Because circulating B lymphocytes do not adhere to fibronectin (Table I), although a fraction of pre-B and B cells from

the bone marrow do (Table II), we conclude that differentiation of bone marrow pre-B cells into B lymphocytes is accompanied by a loss of adhesion to fibronectin. Whether the adhesion of normal bone marrow cells to fibronectin is mediated by the same molecule(s), we characterized in the transformed lymphoid cell lines remains to be established.

Discussion

We have shown that several B lymphoid cell precursor lines, blocked at specific stages of differentiation, specifically adhere to fibronectin in an *in vitro* assay. Whereas Ba F3 cells, presumably a B stem cell (Palacios and Steinmetz, 1985), adhere to the classical cell attachment domain of fibronectin, the B-committed line PD 31 does not. Rather, adhesion of PD 31 cells is mediated by a different region of the fibronectin molecule, which is part of the carboxy-terminal region containing the high-affinity binding site for heparin. An antibody raised against the fibronectin receptor of mouse erythroleukemic cells inhibits lymphoid cell adhesion to fibronectin, and precipitates surface proteins of 140 and 70 kD from surface-radioiodinated Ba F3 cells and of 140 and 120 kD from B-committed cells. Based on this evidence, we propose that these cell lines express different fibronectin receptors that mediate substrate adhesion, and suggest that a receptor with different specificity might be expressed in the course of B cells maturation. Because a large proportion of mouse bone marrow cells of B lineage specifically adhere to fibronectin and to this heparin-binding fragment *in vitro*, we infer that these proteins might play a role in lymphoid precursor cell adhesion to the extracellular matrix.

The fibronectin receptor of Ba F3 cells belongs to the family of receptors that recognize the arg-gly-asp sequence on the cell-binding domain of fibronectin (reviewed by Ruoslahti and Pierschbacher, 1986). Indeed, Ba F3 cells adhere to both intact fibronectin and to its 115-kD chymotryptic fragment that contains the cell binding site (Fig. 3a). Attachment to fibronectin is inhibited by both a monoclonal antibody (N-294) directed against the cell-binding domain of fibronectin and by a heptapeptide containing the arg-gly-asp cell attachment recognition sequence of fibronectin (Fig. 2). They also possess a 140-kD cell surface protein that is specifically bound by the affinity matrix containing the 115 kD cell-binding fragment of fibronectin, and is specifically

Table II. Adhesion of Bone Marrow Cells to Intact Fibronectin and to Its Heparin-binding Fragment

Cell type	Adherent cells		
	Fibronectin	Heparin-binding fragment	BSA
	%		
Total population	48	78	4
B 220 positive cells	55	94	ND
Surface Ig positive cells	26	92	ND

As detailed in Materials and Methods, we prepared a population of mononuclear bone marrow cells depleted of monocytes/macrophages. These were assayed for attachment to fibronectin (20 $\mu\text{g}/\text{cm}^2$), to its carboxy-terminal heparin-binding fragment (5 $\mu\text{g}/\text{cm}^2$), or to BSA (2%) on 60-mm diameter plastic petri dishes. The attached and unattached populations were then stained with either anti-B220 or anti-Ig antibodies. Of the cells used in this assay 49% were B220 positive and 20% were positive for surface Ig. ND, not determined.

eluted by a synthetic peptide containing the classical arg-gly-asp sequence (Fig. 5 *a*). This protein has, therefore, all of the properties of a cell surface fibronectin receptor.

At variance from MEL and Ba F3 cells, the B lineage-committed PD 31 cells adhere to intact fibronectin but not to its 115-kD chymotryptic fragment that contains the classical cell-binding site of fibronectin (Fig. 3 *b*). Accordingly, they have no specific surface protein that could be eluted from an affinity matrix containing the 115-kD chymotryptic fragment of fibronectin (Fig. 5 *b*). PD 31 cells do adhere to chymotryptic fragments of fibronectin purified by affinity chromatography on heparin-Sepharose (Fig. 4 *a*); adhesion is not inhibited either by a 20-fold excess of heparin (Fig. 4 *b*) or the fibronectin monoclonal antibodies N-294 or N-296 (Fig. 4 *c*). Although the heparin-binding preparation is not purified to homogeneity, we are confident that adhesion of PD 31 cells to these peptides does not involve interactions with the classical arg-gly-asp sequence, and is probably not mediated by cell surface-associated heparan sulphate proteoglycans. Thus, we conclude that one or more surface molecules on PD 31 cells are receptors that bind to a different region of fibronectin.

Adhesion to the heparin-binding domain of fibronectin is not restricted to PD 31 cells, in that these heparin-binding fragments of fibronectin also promote adhesion of Ba F3, MEL cells, and normal rat kidney fibroblasts (data not shown). Thus, even cells with a classical fibronectin receptor recognize, in addition, a different region of the fibronectin molecule. These data are consistent with recent evidence that the heparin-binding region of the fibronectin molecule mediates important biologic effects both in fibroblasts (Woods et al., 1986) and in melanoma cells (McCarthy et al., 1986).

Indirect evidence for a receptor-mediated process in the interaction of lymphoid precursor cells with fibronectin comes from the studies with an antiserum raised against the erythroid fibronectin receptor. Immunoglobulins in this antiserum, but not in preimmune serum, block adhesion of MEL and Ba F3 cells to fibronectin-coated wells (Fig. 6, *a* and *b*). The same antibody immunoprecipitates a major protein of ~140 kD from both a total cell extract or an affinity-purified fibronectin receptor preparation from surface radioiodinated MEL cells (Fig. 7), proving that it specifically recognizes the fibronectin receptor. A similar experiment with Ba F3 cells gave identical results (not shown), suggesting that the surface molecules recognized by this antibody in cells of B lineage define their receptor for fibronectin. Inasmuch as the antiserum against the erythroid fibronectin receptor also inhibits attachment of PD 31 cells to intact fibronectin (Fig. 6 *c*) and immunoprecipitates two major polypeptides of 140 and 120 kD from surface-labeled PD 31 and 38 B9 cells (Figs. 8 and 9), we suspect that these polypeptides are the fibronectin receptor(s) of these B-committed lymphoid lines and that they are immunologically cross-reactive with but not identical to the erythroid and Ba F3 cell receptors. Some support for our hypothesis comes from the results obtained with the mouse plasmacytoma line MPC 11. These cells do not adhere to fibronectin (Table I and Fig. 1), and bear little, if any, surface proteins that are specifically immunoprecipitated by the antiserum against the fibronectin receptor (Fig. 8). Whether our inability to detect a species cross-reacting with a fibronectin receptor in MPC 11 cells

reflects a property of the normal plasma cell or is rather linked to neoplastic transformation remains to be established.

Increasing evidence suggests that the "fibronectin receptors" are members of a large family of related but different proteins mediating cell interactions with several extracellular components. Thus, the platelet IIb/IIIa glycoprotein complex, which mediates platelet interaction with fibronectin (Ginsberg et al., 1985), vitronectin (Pytela et al., 1986), fibrinogen (Bennett and Vilaire, 1982), and von Willebrandt factor (Ruggeri et al., 1982), shares antigenic determinants with the fibronectin receptor of neutrophils and monocytes (Burns et al., 1986) and with endothelial cell membrane proteins (Fitzgerald et al., 1985; Thiagarajan et al., 1985). These homologies have recently been found to extend to two leukocyte surface glycoproteins, Mac-1 (Springer et al., 1979) and lymphocyte function-associated antigen-1 (LFA-1) (Davignon et al., 1981), which are encoded by the same 20-kb genomic clone encoding platelet IIb/IIIa (Cosgrove et al., 1986). These surface proteins consist of at least two subunits that undergo characteristic changes of mobility on SDS-PAGE, depending on whether reducing or nonreducing conditions are used. Under nonreducing conditions, indeed, the larger subunit migrates at a slower rate while the smaller subunit migrates faster (see, e.g., Pytela et al., 1985, 1986; Burns et al., 1986; Patel and Lodish, 1986). This is most likely due to the presence of intrachain disulfide bonding in cysteine-rich regions of the molecule, as convincingly deduced from the cDNA sequence of the smaller subunit of the chicken fibroblast protein complex, integrin (Tamkun et al., 1986). In the case of the lymphoid surface molecules characterized here, under nonreducing conditions we could not detect an increased mobility on SDS-PAGE analysis (Fig. 9). Whether this finding is due to a structural difference of the lymphoid receptor(s) remains to be established.

B220 is an early B lineage-restricted antigen defined by the monoclonal antibody RA3-6B2 (Coffman, 1982). About 50% of the B220 positive cells of a mouse bone marrow preparation, enriched in lymphoid precursors, exhibit specific attachment to fibronectin and a even higher percentage adheres to a heparin-binding fragment of fibronectin *in vitro* (Table II). This is also the case with surface Ig-positive cells of the bone marrow (Table II). We do not know whether increased adhesion of lymphoid cells to the heparin-binding fragments of fibronectin reflects an altered conformation of the fibronectin molecule caused by the chymotrypsin treatment or a specific, developmentally regulated event. This question can be addressed experimentally. The function of multiple fibronectin receptors with different specificities, which are apparently expressed at different stages of B cell differentiation, might be involved in the migration of cells within the bone marrow. Expression of fibronectin receptors on lymphoid cells might also serve to anchor these cells to the fibronectin matrix of the bone marrow and prevent their premature release into the bloodstream. Because, however, peripheral blood lymphocytes do not adhere to fibronectin (Table I), surface fibronectin receptors of pre-B cells must be lost or modified at the terminal stages of development. It will be of interest to determine whether the released cells reacquire their ability to adhere to fibronectin in the process of homing into the peripheral lymphoid organs.

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