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Immunology

T-lymphocyte differentiation and the extracellular matrix: Identification of a thymocyte subset that attaches specifically to fibronectin

(thymocyte adhesion/thymus)

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ABSTRACT **A population of murine thymocytes adheres specifically to fibronectin but not to vitronectin, laminin, or collagen type I. The interaction of these thymocytes with fibronectin could be inhibited by the synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro, which comprises the previously identified cell-attachment determinant of the molecule, suggesting that the cell attachment site on fibronectin is recognized by these cells. A similar peptide, in which the aspartate residue had been replaced with glutamate, had no effect on this adhesion. The fibronectin-adherent thymocytes were found to be cortisone-sensitive; to bind peanut agglutinin; to have a Thy-1.2$^+$, Ia$^-$ surface phenotype; and to express H-2 antigen only weakly on their surface. In addition, approximately 80% of the fibronectin-adherent cells expressed L3T4 and 80% expressed Ly-1 on their surface, whereas >95% were positive for Ly-2. The data suggest that these cells, which constitute 10% of all thymic lymphocytes, are cortical thymocytes. We propose that their adhesion to fibronectin may be important for their differentiation. The binding to fibronectin provides a means to selectively isolate these cells for study.**

The ability of an animal to mount an effective immune response against most invading antigens depends upon a functioning complement of cells that have been "educated" in the thymus—that is, T lymphocytes (1). Many of the cell surface antigens that appear unique to T lymphocytes (2, 3) become expressed as T cells differentiate in the thymus. However, the exact nature of the stimuli responsible for this maturation has not been established (4–6). Much work (reviewed in refs. 7 and 8) has shown that (*i*) entrance of precursor T cells into the thymus and their subsequent exit into the circulation are prerequisite events for development of mature T-lymphocyte characteristics, (*ii*) interaction with the thymic epithelium is probably involved in this development, and (*iii*) soluble factors from the thymus are not sufficient to stimulate expression of mature T-cell properties even though they may be necessary. The full complement of molecules involved in this process has not been identified.

The structure of the thymus has been well studied (9–11), and a variety of cell surface antigens have been used to map the location of lymphoid subsets found within distinct regions of the thymus. However, very little has been reported about the physical interaction of thymocytes with their extracellular surroundings. Positional cues for developing thymocytes may come from neighboring cell surfaces (12) or from the extracellular matrix. The extracellular matrix is composed of a number of interwoven macromolecules, some of which have been isolated and shown to support the attachment of various types of cells *in vitro*. Among these are fibronectin (13–17), vitronectin (18–21), the collagens (22–24), and laminin (25–27). Of these molecules, fibronectin and vitronectin have the amino acid sequence -Arg-Gly-Asp- in common (28–34), and this sequence is recognized by two distinct cell surface receptors for these molecules (32–36).

We have found that several lymphoma cell lines recognize the cell attachment site in fibronectin (see below) and that large deposits of fibronectin are present around the vessels and ducts of the thymus (unpublished data). These observations led us to investigate the possible interaction of thymic lymphocytes with this molecule. We report here that a population of thymocytes exists that specifically recognizes and adheres to the -Arg-Gly-Asp- sequence in fibronectin but not to that of vitronectin. The selective expression on thymocytes of specific receptors for fibronectin or other, as yet unidentified adhesive molecules may be an important event in their differentiation process.

MATERIALS AND METHODS

Animals and Cells. The BALB/cByJ mice used were bred in the animal facility at the La Jolla Cancer Research Foundation and were 4–6 weeks old. The lymphoma cell lines tested were the generous gift of W. C. Raschke at this Foundation.

Proteins and Peptides. Fibronectin and vitronectin were prepared from mouse plasma according to Engvall and Rouslahti (37) and Barnes and Silnutzer (38), respectively. Collagen type I was purchased from Sigma, and laminin was purchased from Bethesda Research Laboratories. Peptides were synthesized to our specifications by Bachem Fine Chemicals (Torrance, CA). On the day of the experiment, peptides were dissolved in Dulbecco's modified Eagle's medium (DMEM) and the pH was adjusted to 7.2 with sodium bicarbonate.

Cell Attachment Assay. Microwell plates (96-well, Linbro, McLean, VA) were coated with the designated proteins by incubating the protein solutions at various concentrations in the plates overnight at 4°C. Unbound proteins were removed from the plates by washing with phosphate-buffered saline (PBS, 150 mM NaCl/10 mM sodium phosphate, pH 7.4). The plates were then incubated with DMEM (0.1 ml per well) containing bovine serum albumin (BSA; 2.5 mg/ml; Sigma) for 2 hr at 37°C. That the wells contained graded amounts of functional immobilized protein was confirmed in all cases by independent assay of fibroblast attachment and ELISA (39), though the actual amount of protein bound to the plate was not determined.

Thymocytes were isolated according to published procedure (40). After washing, the thymocytes were resuspended (2×10^7 per ml) in DMEM plus BSA (2.5 mg/ml) for assay of attachment. Thymocytes (100 μl) were added to the protein-coated wells, and the plates were incubated at room

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Abbreviations: PnA, peanut agglutinin; BSA, bovine serum albumin.

temperature for 2.5 hr on an orbital shaker at 60 rpm (these conditions were determined to be optimal). Attached cells were then fixed with 3% paraformaldehyde and stained with 0.5% toluidine blue in 3.7% formaldehyde. Attached cells were counted with an Artek cell counter (Dynatech, Alexandria, VA), or the optical density at 600 nm of amido black (Sigma)-stained cells was determined using the Titertek Multiskan (Flow Laboratories) vertical-pathway spectrophotometer to quantitate attachment.

Cell Separation Based on Peanut Agglutinin (PnA) Binding. PnA-binding cells were obtained by a modification of a described technique (41). Petri dishes (6 cm, Falcon) were coated with PnA (20 μg/ml; Vector Laboratories, Burlingame, CA) dissolved in 0.15 M NaCl/0.05 M Tris Cl, pH 9.5. Thymocyte suspensions (6×10^7 cells) in DMEM containing BSA (2.5 mg/ml) were then added to each Petri plate and incubated at 4°C for 90 min. Unbound cells (PnA^-) were collected by two washes in PBS, and adherent cells (PnA^+) were eluted in 5 ml of 200 mM D(+)-galactose (Sigma). The PnA^+ or PnA^- cells were then evaluated for their ability to attach to substrates.

Cortisone-Resistant Thymocytes. Thymocytes were isolated from mice 48 hr after intraperitoneal injection of 2.5 mg of hydrocortisone acetate (Merck Sharp & Dohme, West Point, PA). Cell recovery was 5–10% of that obtained from saline-treated control animals.

Immunofluorescence. Thymocytes were plated in 8-well Lab-Tek chambers (Miles Sci., Naperville, IL) that had been coated with BSA or fibronectin. After a 2.5-hr incubation, adherent cells were fixed with 1% or 3% paraformaldehyde in PBS for 30 min at room temperature. The antibodies were then added for 1 hr at room temperature, and a rhodamine-conjugated second antibody was added for an additional 60 min. The antibodies used for staining were anti-Ly-2.2 (42), anti-I-A^d (43), anti-H-$2K^dD^d$ (44), anti-Ly-1 (42), and anti-L3T4 (45) (American Type Culture Collection) and anti-Thy-1.2 (42) (Bioproducts for Science, Indianapolis, IN). In some cases, a third antibody, conjugated with rhodamine, was also used to enhance staining. Fluorescent PnA was purchased from Vector Laboratories.

Since variability is observed among laboratories when using different antibodies to quantitate cell surface antigens, we stained whole thymocytes with the antibodies used in this study. We found that thymocytes were 95%, 90%, 96%, 90%, and 95% positive for Ly-1, Ly-2, L3T4, PnA, and Thy-1.2, respectively. Approximately 10% expressed high levels of H-2 antigen on their surface.

RESULTS

Interaction of Lymphoma Cell Lines with Extracellular Matrix Molecules. When a number of lymphoma cell lines of B- and T-cell origin were tested for their ability to adhere to various extracellular matrix molecules, an interesting pattern emerged. Some cells were nonadherent on any of the proteins tested, whereas others bound to one or more of the substrates (Table 1). This observation led us to question whether normal lymphocytes might in some instances also express receptors for one or more extracellular matrix molecules.

Thymocyte Interaction with Known Adhesive Extracellular Matrix Molecules. Isolated thymocytes were incubated on substrates coated with various concentrations of fibronectin, vitronectin, laminin, collagen type I, or BSA (control). Increasing concentrations of fibronectin on the substrate led to a concomitant increase in the number of thymocytes attached per well, until a plateau was reached at about 10 μg of fibronectin/ml of coating buffer (Fig. 1). In contrast, substrates carrying the other proteins did not support the adhesion of thymic lymphocytes, though some adherent macrophages were always observed. Transfer of nonattached cells to a second fibronectin-coated dish showed that the fibronectin-adherent cells had been quantitatively removed in the first incubation. Attached cells were quantitated as described in *Materials and Methods*. Such cells accounted for approximately 10 ± 1% of the total number of thymocytes.

To further assess the specificity of the adhesion of this population of thymocytes to fibronectin, thymocytes were incubated on fibronectin-coated substrates in the presence of a peptide having the sequence Gly-Arg-Gly-Asp-Ser-Pro, the demonstrated cell attachment site of fibronectin (30, 31, 33). This peptide inhibits attachment of a number of cell lines (33). This peptide significantly inhibited the binding of thymocytes to fibronectin (Fig. 2). The peptide Gly-Arg-Gly-Glu-Ser-Pro, which does not have cell attachment-promoting activity (31), had no effect on the ability of thymocytes to bind fibronectin. This result strongly suggests that this population of thymocytes binds specifically to fibronectin and that the region on the molecule recognized by these cells is the same as that used by other cell types (35).

Identification of the Fibronectin-Adherent Thymocyte Subpopulation. Having established that some thymocytes attach to fibronectin, we next wanted to investigate which population of thymocytes was involved in this adhesion. To determine whether the fibronectin-adherent cells were PnA^+ (46), we selected for PnA-adherent cells as outlined in *Materials and Methods*. The data show that 11% of the PnA^+ thymocytes bound to fibronectin (Fig. 3). On the other hand, PnA^- thymocytes showed no binding to fibronectin. The small increase in absorbance observed with these PnA^- cells on fibronectin, as opposed to BSA, was attributable by microscopic inspection to the presence of a small number of macrophages, which have been shown to bind fibronectin

Table 1. Attachment of some representative lymphoma cells to various substrates

	Attachment†					
	T-cell origin			B-cell origin		
Coating*	WR 16.1	WR 2.3	Thy 16.1	RAW 309.1	GD36A	RAW 112.2
Fibronectin	+	−	−	+	++	−
Vitronectin	−	−	−	+	−	−
Type I collagen	−	−	−	−	−	−
Type IV collagen	−	−	−	+	+	−
Laminin	−	−	−	−	−	−
BSA	−	−	−	−	−	−
None	−	−	−	−	−	−

*Wells were coated with the proteins at a concentration of 10 μg/ml. Fibronectin and vitronectin were prepared in our laboratory from human plasma. Collagen and laminin preparations were provided by E. Engvall (La Jolla Cancer Research Foundation). BSA was obtained from Sigma.

†Attachment is indicated as no cells attached (−), 50–95% of the cells attached (+), or >95% of the cells attached (++) during a 1-hr incubation at 37°C.

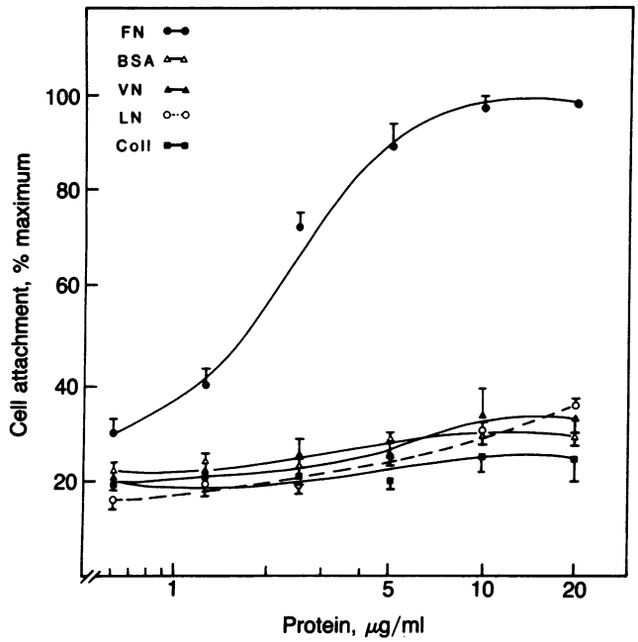

FIG. 1. Adhesion of thymocytes to fibronectin. Thymocytes (2×10^6 per well) suspended in DMEM containing BSA (2.5 mg/ml) were incubated at 24°C for 2.5 hr in wells that had been coated with the indicated concentrations of protein. Maximal attachment of thymocytes to fibronectin was set at 100%, and the adhesion to other proteins is expressed relative to that value. FN, fibronectin; BSA, bovine serum albumin; VN, vitronectin; LN, laminin; Coll, collagen type I. Mean ± SEM are expressed per point ($n = 9$).

(47, 48), and dendritic cells. To confirm that the fibronectin-adherent lymphocytes were indeed PnA^+, rhodamine-conjugated PnA was used to fluorescently stain the cells. All of the lymphocytes that attached to fibronectin were brightly stained by this procedure (data not shown). By use of a rhodamine-conjugated second antibody, the fibronectin-adherent lymphocytes were also found to be positive for Thy-1.2 and weakly positive for H-2 antigen but negative for Ia. Approximately 80% expressed L3T4; 80%, Ly-1; and ≥95%, Ly-2.2 (Fig. 4).

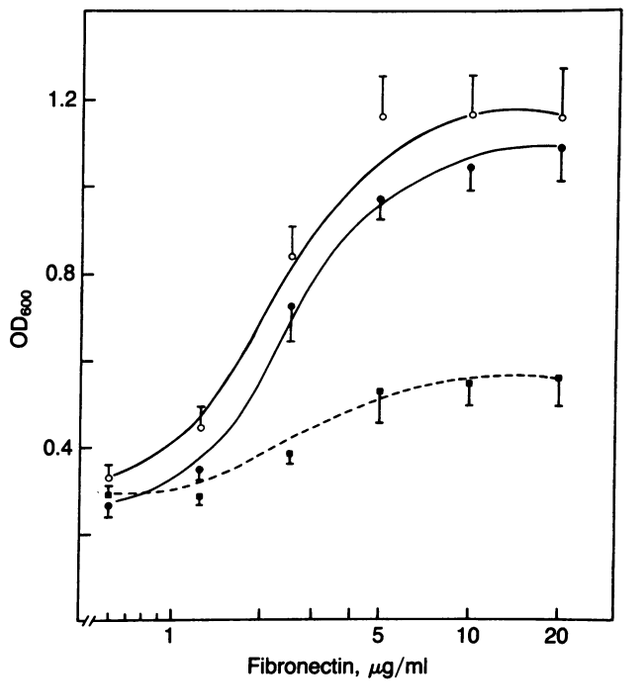

FIG. 2. Inhibition of attachment of thymocytes to immobilized fibronectin by a synthetic fibronectin peptide. Thymocyte adhesion to wells coated with fibronectin at various concentrations was assessed following a 2.5-hr incubation in the absence (●) or presence of Gly-Arg-Gly-Asp-Ser-Pro (■) or Gly-Arg-Gly-Glu-Ser-Pro (○) at 1 mg/ml. OD_{600} of adherent cells stained with amido black was measured. Each point represents the mean ± SEM ($n = 10$).

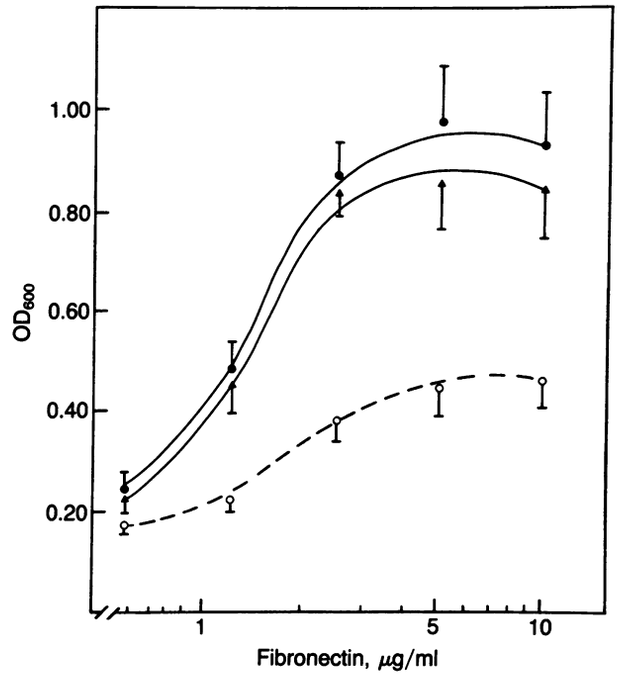

FIG. 3. Attachment of PnA^+ and PnA^- thymocytes to fibronectin. Thymocytes were incubated at 4°C for 90 min on PnA-coated dishes. Cells that did not bind to PnA (PnA^-) and PnA-bound cells that were eluted with 200 mM D(+)-galactose (PnA^+) were collected and the attachment of these subsets to fibronectin was assessed. The fibronectin-adherent cells were quantitated and are expressed as mean ± SEM ($n = 9$). ●, PnA^+ thymocytes; ○, PnA^- thymocytes; ▲, unfractionated thymocytes.

These data suggested that the fibronectin-adherent cell population are cortical thymocytes. To test this possibility further, thymocytes were isolated from corticosteroid-treated animals. Thymocytes from these animals displayed no detectable binding to fibronectin (data not shown), suggesting that at least the vast majority of the fibronectin-adherent cells originated in the thymic cortex (7).

Careful microscopic evaluation of the adherent cells revealed that a number of thymocyte rosettes also bound to fibronectin-coated dishes. These thymocyte rosettes have been shown to contain cells with a surface-antigen phenotype that is typical of immature thymocytes (12). At this point we do not know whether it is the central macrophage-like cell or the rosetted lymphocytes which makes the contact with the substrate. These structures, however, constitute only a small fraction of the adherent population.

DISCUSSION

We have demonstrated that ≈10% of murine thymocytes bind to the extracellular matrix glycoprotein fibronectin. This adhesion to fibronectin is specific, as it was inhibited by a peptide comprising the cell-binding site of fibronectin, Gly-Arg-Gly-Asp-Ser-Pro (30, 31). Also, other known adhesive glycoproteins such as laminin, vitronectin, and collagen type I did not mediate thymocyte binding.

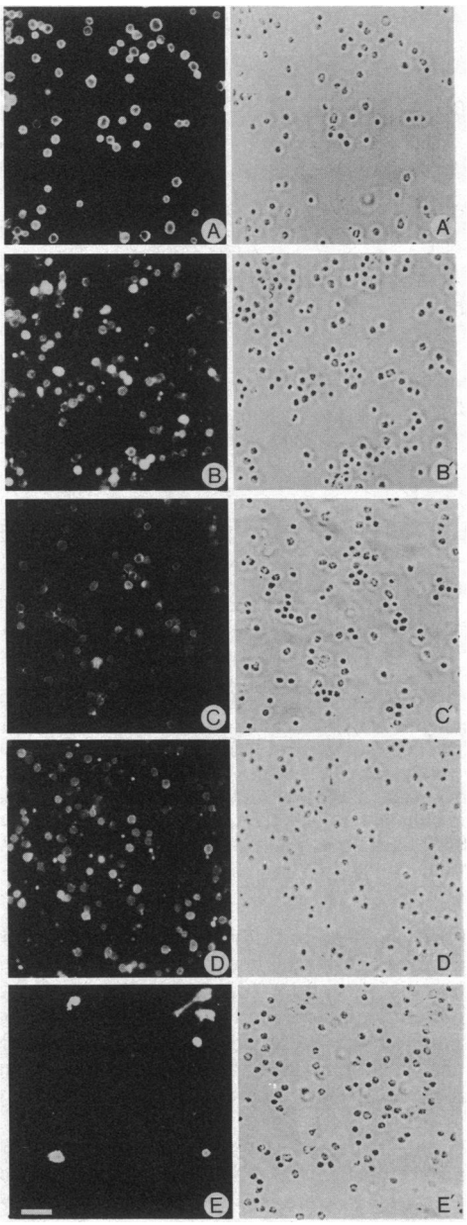

FIG. 4. Localization of surface antigens on fibronectin-adherent thymocytes, as determined by immunofluorescence. The localization of Thy-1 (*A*), Ly-1 (*B*), Ly-2 (*C*), L3T4 (*D*), and Ia (*E*) was determined on fibronectin-adherent thymocytes. The same field under phase-contrast is shown for each in *A'*, *B'*, *C'*, *D'*, and *E'*. (Bar = 20 μm.)

The fibronectin-adherent thymocyte population is PnA^+, Thy-1.2^+, H-2-poor, Ia^-, and cortisone-sensitive. The majority also expressed L3T4, Ly-1, and Ly-2.2. Taken together, these data indicate that these cells were derived from the thymic cortex (11).

Recently, Scollay and Shortman (49) have proposed a pathway for thymocyte development, which was based on phenotypic similarities and intrathymic positions of the various thymocyte subsets. They found that a majority of cortical thymocytes, which represent 80% of the total thymocyte population, are Thy-1^+, Ly-1^+, Ly-2^+, and L3T4^+. The cell surface antigens expressed by the fibronectin-adherent cells we have identified make it likely that they are, at least in part, a subset of this cortical population.

The port of entry for stem cells into the thymus proper is the capillary endothelium and the underlying connective tissue. The selective adherence of T and B lymphocytes to glycoproteins on high endothelial venules of peripheral lymphoid tissue has been demonstrated (50, 51). This binding appears necessary for the migration of recirculating lymphocytes from the blood into lymph nodes and Peyer's patches (52, 53). Similar types of interactions may be involved in the migration of stem cells into the thymus. Within the thymus, the hemopoietic stem cell is influenced to differentiate by the microenvironment found there. It appears that the nascent stem cell enters the thymic cortex and, as it develops, moves toward the medulla (54–56). This orchestrated movement must be a response to molecules in the environment of the cell and may, at least in part, represent the making and breaking of adhesive contacts. The idea has also been advanced that cortical and medullary thymocytes belong to separate populations (8, 56, 57). If that is the case, one must ask what recognition capabilities would dictate such a separation.

That a group of thymocytes can bind to fibronectin suggests that they possess fibronectin receptors. These receptors may actually endow the lymphocytes with the ability to traverse connective tissue during entrance into or exit from the thymus (58). It is, however, presently unknown if this fibronectin-adherent thymocyte population will exit the thymus as mature T cells, because the question of whether peripheral T lymphocytes are derived from medullary (59) or cortical (60, 61) thymocytes remains unresolved. Alternatively, the fibronectin receptors may coordinate movement of cells within the thymus.

On the other hand, the interaction with fibronectin may, in itself, constitute a differentiative signal. Though we currently have no data concerning the influence of fibronectin on lymphocyte development, several investigators have presented experiments that implicate fibronectin in differentiative processes. Pennypacker *et al.* (62) suggested, for example, that fibronectin synthesis or accumulation may be an important mechanism for regulating chondrogenesis. Boucaut *et al.* (63) presented evidence that suggests the involvement of adhesive receptors in embryonal morphogenesis. Indeed, the controlling effects of extracellular environment on cell shape, cell survival and growth, and maintenance of the differentiated state of cells have been described (64–67). Thus, it is reasonable to suspect that developing lymphocytes, at times when they exist as sessile populations (e.g., in the thymus, in the bone marrow, and in the peripheral lymphoid tissue), may attach to and be influenced by adhesive molecules in these locations.

Our data establish the existence of lymphocytic cells within the thymus that interact with an extracellular matrix component, fibronectin, and point toward the possible existence of other such interactions. They also offer a new probe for better understanding T-lymphocyte differentiation by providing a means to isolate a population of thymocytes from the thymic cortex based on a specific functional property. The possible interaction of other populations of thymocytes with adhesive molecules that may be as yet unidentified, and the effects of the adhesion of the identified thymocyte population to fibronectin, can now be investigated.

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