ACTIVATION OF CD4 CELLS BY FIBRONECTIN AND ANTI-CD3 ANTIBODY

A Synergistic Effect Mediated by the VLA-5 Fibronectin Receptor Complex

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It has become evident that the CD4 subset of lymphocytes is a heterogeneous collection of cells with multiple phenotypes and functions (1–3). Recently, $CD4^+$ lymphocytes have been subdivided into reciprocal subsets based on their expression of unique isoforms of the leucocyte common antigen (LCA)[1]/T200 family of antigens (CD45/CD45R) (4–6) and the CDw29 (4B4) antigen (7). $CD4^+CDw29^+$ cells (in fact, $CDw29^{high}$) provide strong helper function for B cell Ig production, respond maximally to recall antigens (7), and induce CD8 cells to exert class I MHC-restricted cytotoxicity (8). The $CD4^+CD45R^+CDw29^-$ (in fact, $CDw29^{low}$) cells, in contrast, can induce CD8 cells to suppress Ig synthesis but cannot provide helper function, or respond to recall antigens. Anti-2H4(CD45RA) antibody has been shown to define the 200-kD and 220-kD isoforms of the LCA/T200 family of antigens (9), whereas UCHL-1 (CD45RO) defines the 180-kD isoform of this family (10, 11). Anti-4B4(CDw29) antibody has been shown to be reactive with the VLA/integrin antigen family (9, 12, 13), which is comprised of a common β chain noncovalently complexed to distinct α chains (13, 14).

The integrin family of adhesion molecules has been shown to play an important role in the function of mammalian cells (15). Members of this family include CD11a-(LFA-1), CD11b(Mol), p150-95 (16, 17), and platelet glycoprotein IIb/IIIa (18, 19), as well as receptors for extracellular matrix proteins such as fibronectin, collagen, and laminin (15, 20–22). Each of the α and β subunits of the integrin family have

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[1] *Abbreviation used in this paper:* LCA, leukocyte common antigen.

been reported to be structurally related (23–26), and many of these receptors recognize an Arg-Gly-Asp(RGD) sequence (21, 27). Both LFA-1 and Mol, acting as adhesion structures, play an important role in lymphoid function (17).

In contrast, the functional role of fibronectin, laminin, and collagen receptors in the immune response has yet to be established. Both a subset of murine thymocytes and a T cell lymphoma cell have been shown to bind to fibronectin, and this binding was inhibited by the peptide sequence Gly-Arg-Gly-Asp-Ser-Pro (28, 29). Savagner et al. (30) have shown that invasive avian lymphocyte precursors contain integrin/fibronectin receptors, and that their invasiveness could be blocked by inhibitors of fibronectin receptor interaction. Moreover, it has been suggested that members of the VLA/integrin family of antigens can act as fibronectin receptors on lymphocytes (31).

Given that our earlier studies showed that $CD4^+CDw29^+$ cells and $CD4^+CDw29^-$ cells were functionally distinct (7), we examined the role of the VLA/fibronectin receptor family as defined by anti-4B4(CDw29) and related antibodies in CD4 T cell function. In the present study, we show that under appropriate culture condition, a mixture of anti-CD3 antibody (32) and fibronectin can induce the activation of CD4 cells, whereas neither alone can do it. Our studies suggest that fibronectin may contribute to CD4 cell activation through the CD3-TCR complex under physiologic conditions (32). In addition, anti-CDw29 (4B4) antibody blocked the activation of CD4 cells in this system. Lastly, through the use of a new antifibronectin receptor antibody, we have shown that the VLA-5 protein acts as functional fibronectin receptor on CD4 cells and that this VLA-5 receptor is preferentially expressed on the $CD4^+CDw29^+$ subset of cells and may account for the functionally unique program of these cells.

Materials and Methods

Reagents. Human fibronectin and laminin were obtained from Collaborative Research, Lexington, MA. Type I, III, and IV collagen from human placenta, phorbol 12-myristate, 13-acetate, L-leucine methylester, aprotinin, Cowan strain 1 bacteria, and PMSF were from Sigma Chemical Co., St. Louis, MO. The GRGDSP peptides were from Peninsula Laboratories, Inc., Belmont, CA. For use in the serum-free medium, BSA, transferrin, and soybean lipids were from Boehringer Mannheim Biochemicals, FRG. Iscove's modified Dulbecco's medium was from Sigma Chemical Co. rIL-2 was from Biogen, Geneva, Switzerland.

Preparation of Fibronectin Fragments. Fibronectin fragments (Fig. 4, fractions A, B, C, D, and E), the kind gift of Dr. Joyce K. Czop (Brigham and Women's Hospital, Harvard Medical School, Boston, MA), were prepared as described previously (33, 34). Each of the fractions was analyzed for fragments bearing fibronectin epitopes by immunoblotting using five mouse antifibronectin mAbs (33): BC7, which recognizes an epitope within the 27,000-mol wt NH_2 terminus; CE9, which recognizes an epitope within the 18,000-mol wt link region between the gelatin-binding and cell-adhesive domains; BD4, which recognizes an epitope within the cell-adhesive domain; AB3, which recognizes an epitope on the α chain between the high affinity heparin-binding domain and the interchain disulfide bonds; and CPG1, which recognizes an epitope adjacent to the disulfide bonds near the COOH terminus. Briefly, intact plasma fibronectin was isolated from the cryoprecipitate fraction of fresh, frozen, heat-inactivated normal human plasma by sequential chromatography on Sepharose 4B and gelatin-Sepharose columns. Intact fibronectin was treated with chymotrypsin and chromatographed on gelatin-Sepharose. Fraction A contains the non-gelatin-binding fragments and fraction B contains the gelatin-binding fragments. Fraction C was prepared from a cathepsin D digest of intact plasma fibronectin by sequential affinity chromatography on gelatin-Sepharose, monoclonal CPG1 anti-fibronectin-Sepharose, and monoclonal CE9 anti-fibronectin-Sepharose.

The non-gelatin-binding fragments that did not bind CPG1-Sepharose were eluted from CE9-Sepharose with 0.1 M glycine-HCl, pH 2.5. Immunoblotting revealed the major protein in fraction C to be a 130,000-mol wt fibronectin fragment bearing both the CE9 and BD4 epitopes. Intact fibronectin treated with cathepsin D was also sequentially chromatographed on gelatin-Sepharose, heparin-Sepharose, and CE9-Sepharose. Fraction D contains the non-gelatin-binding fragments eluted from heparin-Sepharose with 0.5 M NaCl; fraction E contains the non-gelatin-binding fragments eluted from heparin-Sepharose with 0.1 M NaCl that did not bind CE9-Sepharose. The 110,000-mol wt fibronectin cell-binding fragment (fraction F) was purified as described (35). Immunoblotting confirmed that this 110,000-mol wt fragment contained the BD4 epitope but not the CE9, BC7, AB3, or CPG1 epitopes of plasma fibronectin. The functional domain composition and fibronectin epitopes of the major proteins in each of the fractions used in this study are summarized in Fig. 4.

Antibodies. mAbs reactive with the lymphocyte surface antigens, CD2(T11, IgG2b), CD3 (RW24B6, IgG1; OKT3, IgG2a), CD4 (12T44D11, IgG1), CD1(T6, IgG1), CD8(21Thy 2D3, IgG1), CD11a(2F12, IgG1), CD11b(Mol, IgM), CDw29(4B4, IgG1), 8F2(IgG1), and CD45R-(2H4, IgG1), have been previously described (4, 7, 36–38). mAb 16 was an IgG2a of rat origin. Full characterization of this antibody is described elsewhere (38a). mAb L243 directed against common epitopes of the DR antigen was obtained from a cell clone from the American Type Culture Collection, Rockville, MD. Goat anti-mouse Ig antibody was from Tago Inc., Burlingame, CA. Anti-CD45R(2H4) and anti-CDw29(4B4) antibodies conjugated to phycoerythrin were from Coulter Immunology, Hialeah, FL. Rat IgG and rabbit anti-rat Ig antibody and FITC-conjugated rabbit anti-rat Ig antibody were from Jackson Immunoresearch, West Grove, PA. J143 antibody (VLA-3 specific) was a gift from Dr. L. J. Old, Memorial Sloan-Kettering Cancer Center, New York. B-5G10 antibody (VLA-4 specific) was a gift of Dr. M. E. Hemler, Dana-Farber Cancer Institute. Anti-8F2 antibody was developed by standard hybridoma technique after immunization of a mouse with PHA-activated T cells from *Saguinus Oedipus*. It was determined that anti-8F2 reacted with the VLA-4 protein as follows. The α subunit immunoprecipitated by anti-8F2 showed the same molecular weight and isoelectric point as that immunoprecipitated by the B-5G10 antibody, which specifically recognizes the $\alpha 4$ subunit of the VLA-4 molecule (14). Moreover, sequential immunoprecipitation studies showed that both antibodies reacted with the same molecules.

Preparation of Anti-CD3 Antibody-coated Microtiter Plates. 100 μl of protein A-purified anti-CD3 antibody at 100 ng/ml in 0.01 M PBS, pH 7.4, was placed in each well of a 96-well flat-bottomed microtiter plate (3595, Costar, Cambridge, MA), which was then incubated at room temperature for >3 h. After washing twice with PBS, the indicated amounts of extracellular matrix proteins with or without the addition of antibodies directed against different lymphocyte surface antigens were placed in each well and incubated at room temperature for an additional hour.

Preparation of Cells. Human PBL were isolated from healthy donors as described previously (39). Briefly, the lymphocytes were separated into erythrocyte rosette-positive (T cells) and -negative populations with sheep erythrocytes. The T cells were depleted of contaminating monocytes by adherence to plastic plates. Further removal of monocytes from T cells was achieved by incubation with 5 mM L-leucine methyl ester HCl. $CD4^+$ cells were obtained by panning on anti-Ig-coated petri dishes with anti-CD8 antibody to remove CD8 cells, and anti-CD11b and L243 antibodies to remove residual monocytes. The CD4 cells thus obtained were >90% CD4 with <5% contamination by $CD8^+$ cells. The contamination by monocytes was <1 in 500 cells as determined by peroxidase staining. These highly purified CD4 cells were used for all experiments performed in this study.

Cell Culture and Proliferation Assay. CD4 cells were cultured in triplicate in anti-CD3 antibody-coated wells (with or without extracellular matrix proteins) at a concentration of 10^5 cells/well in serum-free medium consisting of Iscove MEM supplemented with 0.1% BSA, 30 μg/ml human transferrin, 10 μg/ml soybean lipids, 50 μg/ml cholesterol, 4 mM L-glutamine, 25 mM Hepes buffer (Microbiological Associates, Bethesda, MD), 0.5% sodium bicarbonate, and 50 μg/ml of gentamicin sulfate (Schering, Kenilworth, NJ). After 4 d in culture, each well was labeled with 1 μCi of [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) for 18 h. Cells were then harvested on a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA), and [^{3}H]TdR incorporation was measured on a scintillation counter.

Analysis of Leukocyte Populations with Single- and Two-color Fluorescence Flow Cytometry. Single- and two-color fluorescence flow cytometric analysis was performed on an EPICS C cell sorter (Coulter Electronics, Hialeah, FL), as described previously (39). Briefly, for single-color analysis, cells were stained with antibody 16 followed by staining with an FITC-conjugated $F(ab')_2$ fragment of rabbit anti-rat Ig antibody. For two-color analysis, cells stained as above were incubated subsequently with 100 μg/ml rat Ig for 15 min. The cells were then stained with phycoerythrin-conjugated anti-CDw29 or anti-CD45R antibodies. Control cell samples were stained with either rat Ig followed by FITC $F(ab')_2$ rabbit anti-rat Ig and/or isotype-matched mouse irrelevant antibodies conjugated to phycoerythrin (Coulter Immunology).

Radiolabeling and Immunoprecipitation of Cell Surface Proteins. CD4 cells (10^7) or 5×10^6 cells from other cell lines (HPB-ALL or K562) were labeled with 1 mCi of ^{125}I by the lactoperoxidase technique, as described previously (40). Labeled cells were solubilized in 0.5% Triton X-100, 10 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl, 1 mM PMSF, and aprotinin (10 μg/ml), and then precleared overnight at 4°C with 1 ml 10% (vol/vol) *Staphylococcus aureus* Cowan strain 1 bacteria. The precleared lysate was then incubated at 4°C for 4 h with antibodies coupled to Sepharose 4B beads. The antibodies, T6, 4B4, 8F2, goat anti-mouse Ig, and rabbit anti-rat Ig, were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) at a concentration of 5 mg/ml, according to the procedure recommended by the manufacturer. Before using for immunoprecipitation, J143 and antibody 16 were incubated with goat anti-mouse Ig and rabbit anti-rat Ig antibodies coupled to Sepharose 4B, respectively. In preclearing experiments, 0.4 ml of cell lysates was precleared five times with 50 μl of 50% suspension of antibody coupled to sepharose 4B. The immunoprecipitates were washed twice with lysis buffer, three times with 0.5% deoxycholate, 10 mM Tris-HCl, 140 mM NaCl, pH 8.2, and once with 10 mM Tris-HCl, pH 8.0. Immune complexes were analyzed under reducing or nonreducing conditions by SDS-PAGE (6%) as described by Laemmli (41) and examined by autoradiography.

Two-dimensional Gel Electrophoresis. Two-dimensional PAGE was carried out according to O'Farrell (42). Briefly, samples were eluted from immunoprecipitates by incubation with 8 M urea, 2% NP-40, 5% 2-ME, and 2% ampholytes (pI 3.5–10; Bio-Rad Laboratories, Richmond, CA). Treated samples were applied to 4% acrylamide tube gels and subjected to IEF for 24 h at 150 V. The gels were then equilibrated with SDS-PAGE diluent buffer containing 2-ME subjected to SDS-PAGE (6%), and then processed as described above for one-dimensional SDS-PAGE.

Results

CD4 Cells Can Be Activated through the Combination of Anti-CD3 Antibody and Extracellular Matrix Proteins. To investigate whether extracellular matrix proteins provide a complementary signal in the activation of CD4 cells when combined with anti-CD3 antibody, we studied the effects of collagen types I, III, and IV, plasma fibronectin, and laminin on the proliferation of highly purified CD4 cells. When purified CD4 cells were cultured with the anti-CD3 antibody in serum-free medium, there was no proliferation above background, using antibody concentrations of up to 5 μg/ml (data not shown). Also, extracellular matrix proteins alone could not induce CD4 cell activation (data not shown). Utilizing this culture system, we then examined the effect of the addition of the different extracellular matrix proteins combined with the anti-CD3 antibodies on the proliferation of CD4 cells (Fig. 1). Collagen types I, III, and IV, and fibronectin, when combined with anti-CD3 antibody, were able to elicit the proliferation of CD4 cells. This was particularly true of fibronectin, which markedly induced CD4 cell proliferation. Laminin, even in the presence of anti-CD3 antibody, had no effect.

Fibronectin Receptors on CD4 Cells Are Included among the Molecules Recognized by Anti-4B4 Antibody, and the RGDS Sequence Is Involved in the Interaction of Fibronectin with These

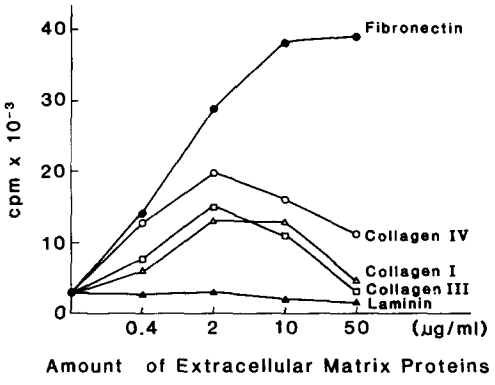

FIGURE 1. Extracellular matrix proteins can induce the proliferation of CD4 cells in combination with anti-CD3 antibody. Flat-bottomed plates were coated with anti-CD3 antibody (100 ng/ml) and/or different extracellular matrix protein at various concentrations as indicated in the figure. CD4 cells were cultured in these plates in serum-free medium for 4 d, at which time proliferation was assessed by determining [^{3}H]thymidine incorporation. The data are expressed as the mean of triplicate samples. Each SE was <15%. The combination of anti-CD3 antibody and type I, III, or IV collagen, or fibronectin induced the proliferation of CD4 cells. When flat-bottomed plates were coated with extracellular matrix proteins alone, [^{3}H]thymidine incorporation was always <100 cpm. The data shown are representative of three separate experiments.

Receptors. Since the above studies showed that the combination of fibronectin and anti-CD3 was a potent activator of CD4 cells, we next focused on the interaction between fibronectin and its receptor in CD4 activation. We examined whether the interaction of fibronectin with an integrin receptor on CD4 cells was required for the observed cell activation. Since anti-4B4 (CDw29) antibody has been shown to react with the common β subunit bound to a number of α subunits of the VLA integrin family (9, 12, 13), we undertook experiments to determine whether or not anti-4B4 could modulate the proliferation of CD4 cells. As shown in Fig. 2, anti-4B4 antibody completely inhibited the proliferative response of CD4 cells activated by anti-CD3 plus fibronectin. In contrast, anti-LFA-1 of the same isotype had no effect. Importantly, anti-4B4 antibody did not inhibit the proliferation of CD4 cells induced by the combination of anti-CD3 antibody and IL-2 or PMA. This observation suggests that anti-4B4 antibody might block the interaction of fibronectin with its receptor on CD4 cells.

Through the use of small synthetic peptides deduced from the primary structure of the fibronectin cell attachment domain, the tetrapeptide Arg-Gly-Asp-Ser (RGDS)

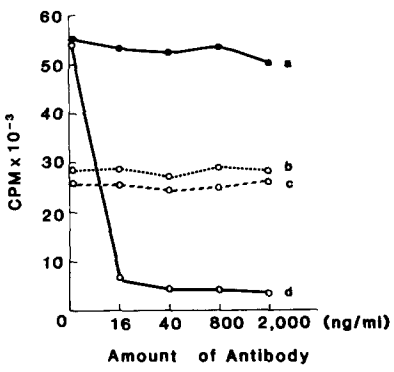

FIGURE 2. Anti-4B4 antibody inhibits the proliferation of CD4 cells induced by the combination of anti-CD3 antibody and fibronectin. CD4 cells were cultured in anti-CD3 antibody and fibronectin-coated plates, to which varying concentrations of LFA-1 antibody (*a*) or 4B4 antibody (*d*) were added. (*b* and *c*) CD4 cells were cultured in anti-CD3 antibody-coated plates with IL-2 (100 U/ml) or PMA (1 ng/ml), respectively, and varying amounts of anti-4B4 antibody were added to each well (*dashed lines*). [^{3}H]thymidine incorporation of CD4 cells stimulated by IL-2 or PMA alone was <1,000 cpm. CD4 cells were cultured in serum-free medium in these plates for 4 d and the proliferation was assessed by determining [^{3}H]thymidine incorporation. The data are expressed as the means of triplicate samples. Each SE was <15%. When flat-bottomed plates were coated with fibronectin alone, the [^{3}H]thymidine incorporation was <100 cpm. The data are representative of three separate experiments.

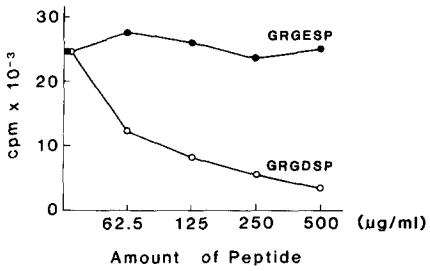

FIGURE 3. RGDS-containing peptide inhibits the proliferation of CD4 cells induced by anti-CD3 antibody and fibronectin. The indicated concentrations of GRGDSP peptide or control GRGESP peptide were added to culture wells where CD4 cells were stimulated by anti-CD3 antibody and fibronectin as described in Fig. 1. GRGDSP peptide inhibited the proliferation in a dose-dependent fashion, whereas GRGESP peptide did not affect proliferation. The data are representative of three separate experiments.

has been shown to be a crucial minimal sequence for cell attachment activity (21, 27). It synergizes with a secondary key site to mediate cell adhesion, spreading, and cytoskeletal effects (43). To investigate the involvement of the RGDS sequence in the interaction of fibronectin with its receptors on CD4 cells, we examined the function of this sequence in our culture system. When the hexapeptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) containing the RGDS sequence was added to cell cultures, the proliferation of CD4 cells in response to anti-CD3 antibody plus fibronectin was inhibited in a dose-dependent fashion, reaching 90% inhibition at a maximum concentration of 500 μg/ml of peptide (Fig. 3). In contrast, no inhibition was observed with the related control hexapeptide GRGESP.

Further support for the involvement of the RGDS sequence in the interaction of fibronectin with lymphocytes was obtained by incubating the cells in anti-CD3 antibody plus proteolytic fragments of fibronectin. Fig. 4 shows the characteristics of the various of well-described proteolytic fragments of fibronectin used in this study. In combination with anti-CD3 antibody, proteolytic fragments containing the cell-adhesive domain and the high affinity heparin-binding domain (Fig. 4 *A*) or the

Fraction	Major Protein(s)	Recognition by				
		BC7	CE9	BD4	AB3	CPG1
Intact	N-▲-□-●-◇-S	+	+	+	+	+
Fibronectin	N-▲-□-●-◇-S	+	+	+	-	+
A	●-◇-S	-	-	+	+	-
	◇-S	-	-	+	-	-
B	□	-	-	-	-	-
	□-●	-	+	-	-	-
C	●-	-	+	+	-	-
D	◇-S	-	-	-	-	-
	◇-S	-	-	-	+	+
E	◇	-	-	+	-	-
	◇	-	-	+	-	-
	S	-	-	-	+	+
F	◇	-	-	+	-	-

FIGURE 4. Graphic representation of the proteolytic fragments of plasma fibronectin used in this study. The major proteins contained within each of the fractions of proteolytic fibronectin fragments are depicted with their functional domains aligned with those of the intact molecule. The regions within the intact molecule are symbolically shown as: NH_2-terminus domain (N-▲); gelatin-binding domain (□); 18,000-dalton link between the gelatin-binding and cell-adhesive domains (●); cell-adhesive domain (-∿∿-); and high affinity heparin-binding domain (◇). A subunit chain difference is depicted by the asterisk above the α chain and its derivatives. The major proteolytic fragments are described in terms of their recognition by five antifibronectin mAbs.

cell-adhesive domain and the adjacent 18,000-dalton peptide link between the gelatin-binding and cell-adhesive domains (Fig. 4 *C*) caused the proliferation of CD4 cells (Fig. 5). A mixture of proteolytic fragments consisting of the cell-adhesive domain alone and the COOH-terminal region of fibronectin containing the interchain disulfide bonds (Fig. 4 *E*) produced virtually no proliferation of CD4 cells in combination with anti-CD3 antibody. Fragments containing the gelatin-binding domain either alone or attached to the 18,000-dalton link between gelatin-binding and cell-adhesive domains (Fig. 4 *B*) or fragments containing the high affinity heparin-binding domain alone or attached to the COOH terminus of plasma fibronectin (Fig. 4 *D*) also could not produce significant proliferation of CD4 cells. In subsequent studies, incubation with a single purified 110,000-dalton cell-adhesive domain fragment containing at least part of the link region between the cell-adhesive and the gelatin-binding domains, but domains lacking heparin-binding sites (Fig. 4 *F*) plus anti-CD3 antibody induced half as much proliferation of CD4 cells as did incubation with intact plasma fibronectin plus anti-CD3 antibody (average of 55% in two experiments). Thus, the cell-adhesive region was necessary, but not fully sufficient, for maximal proliferation.

VLA-5 Complex of the VLA/Integrin Family Acts as a Proliferation-associated Fibronectin Receptor on CD4 Lymphocytes. The different proteins in the VLA family are composed of one common β subunit and one of several different α subunits on the cell surface of lymphocytes (14). Recently, Takada et al. (31) suggested that the VLA-3 and VLA-5 proteins might act as fibronectin receptors on lymphocytes. As shown above, our anti-integrin anti-4B4 (CDw29) antibody blocks the activation of CD4 lymphocytes by the mixture of anti-CD3 and fibronectin. However, immunoprecipitates of anti-4B4 contain common β chain and several α subunits. Thus, it is not clear which of the subunits are actually involved in anti-CD3 fibronectin-induced CD4 cell activation. We tested a newly developed rat anti-human fibronectin receptor antibody, termed antibody 16, that seems to identify on K562 cells the same structures precipitated by anti-4B4 antibody. It should be noted that K562 cells primarily express the VLA-5 protein (31). In fact, neither anti-VLA-3 (J143) nor anti-VLA-4 (8F2) antibodies recognized any proteins from the K562 cell line (data not shown).

As shown in Fig. 6, anti-4B4 immunoprecipitated a broad band (110–130 kD) and several α chains under both nonreducing and reducing conditions (Fig. 6, *a* and

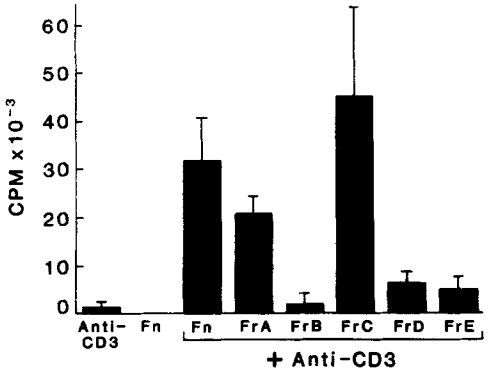

FIGURE 5. Proteolytic fragments containing the cell adhesion domain of fibronectin induce the proliferation of CD4 cells in combination with anti-CD3 antibody. CD4 cells were cultured in plates coated with both anti-CD3 antibody (100 ng/ml) and various proteolytic fragments of fibronectin (10 μg/ml). After culturing for 4 d, the proliferation was assessed by determining [^{3}H]thymidine incorporation by the CD4 cells. These values are the means ± SD of three separate experiments. Proteolytic fragments containing a cell-adhesive domain can induce the proliferation of CD4 cells in combination with anti-CD3 antibody.

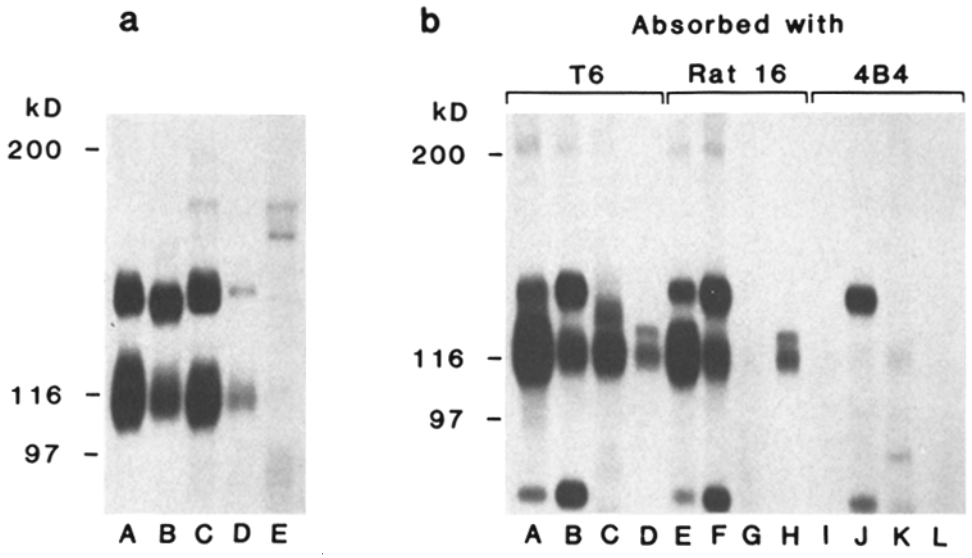

FIGURE 6. (*a*) Comparison of immunoprecipitation by anti-4B4, anti-8F2, and antibody 16. Cell extracts of ^{125}I-radiolabeled HPB-ALL cell line were immunoprecipitated using anti-4B4, anti-8F2, antibody 16, J143 antibody, and rabbit anti-rat Ig antibody (lanes *A*, *B*, *C*, *D*, and *E*, respectively). Samples were analyzed by SDS-PAGE (6% polyacrylamide) under nonreducing conditions. (*b*) Preclearing analysis of cell extracts of ^{125}I-radiolabeled HPB-ALL cell line by anti-4B4 and antibody 16. Cell extracts were precleared with the control antibody anti-T6, antibody 16 (rat 16), and anti-4B4, (lanes *A–D*; *E–H*; and *I–L*, respectively). Cell extracts from the HPB-ALL cell line were immunoprecipitated with anti-4B4, 8F2, 16, and J143 antibodies, (lanes *A*, *E*, *I*; *B*, *F*, *J*; *C*, *G*, *K*; and *D*, *H*, *I*, respectively). Reduced samples were analyzed by SDS-PAGE (6% polycrylamide).

b, lanes *A* and *B*), respectively, from the HPB-ALL cell line. Anti-VLA-4 (8F2) immunoprecipitated 110-kD β and 140-kD α (Fig. 6 *a*, and lane *B*) under nonreducing conditions and 130-kD β and 150-kD α subunits (Fig. 6 *b*, lane *B*) under reducing conditions. Although the α chain recognized by antibody 16 (Fig. 6 *a*, lane *C*) is similar in mobility under nonreducing conditions to the α subunit recognized by the J143 antibody (VLA-3) (Fig. 6 *a*, lane *D*), under reducing conditions, the α subunit recognized by antibody 16 has a higher molecular weight than that recognized by the anti-VLA-3 antibody (Fig. 6 *b*, lanes *C* and *D*).

Furthermore, when cell lysates from the HPB-ALL were incubated with antibody 16, the proteins recognized by J143 antibody (VLA-3) were not cleared (Fig. 6 *b*, lane *H*). However, incubation of cell lysates with anti-4B4 antibody precleared the protein recognized by both J143 and 16 antibodies (Fig. 6 *b*, lanes *K* and *L*). Further evidence that antibody 16 does not crossreact with the VLA-3 α subunit was obtained from two-dimensional gel analysis. The α subunit from HPB-ALL cells recognized by antibody 16 had a more acidic isoelectric point than the β subunit (Fig. 7 *A*), while the VLA-3 α subunit recognized by J143 antibody has a more basic isoelectric point than the β subunit (Fig. 7 *B*). Similarly, when antibody 16 was used to immunoprecipitate cell lysates from the K562 cell line, the α chain once again was more acidic than the β chain (Fig. 7 *D*). Thus, the proteins recognized by antibody 16 are different from VLA-4 (Fig. 7 *C*) and VLA-3 (Fig. 7 *B*). Finally, antibody

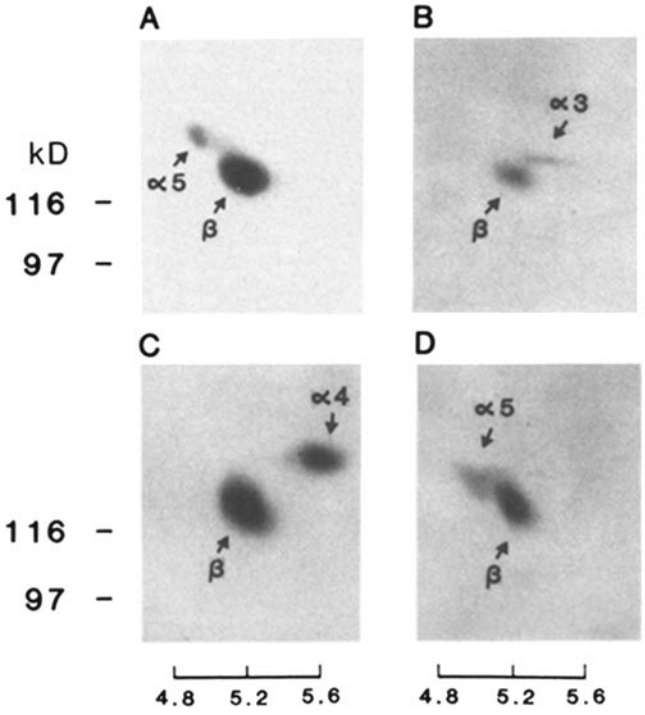

FIGURE 7. Two-dimensional gel analysis of immunoprecipitates by 16, 8F2, and J143 antibody using the HPB-ALL cell line. ^{125}I-radiolabeled cell extract from HPB-ALL cells was immunoprecipitated with antibody 16 (*A*), J143 antibody (*B*), and 8F2 antibody (*C*). An ^{125}I-radiolabeled cell extract of the K562 cell line was immunoprecipitated with antibody 16 (*D*). α chains of the immunoprecipitates with antibody 16 from HPB-ALL and the K562 cell line migrated to a more acidic point than the β chain, whereas those immunoprecipitated by J143 and 8F2 antibodies migrated to a point more basic than the β chain.

16 did not crossreact with the α subunit of the VLA-1 and VLA-2 proteins that have been shown to be expressed on activated T lymphocytes (data not shown). The above results indicate that antibody 16 is specific for the VLA-5 protein.

We used these mAbs to investigate which members of the VLA family within the 4B4 molecules are actually involved in the synergistic activation of CD4 cells by anti-CD3 and fibronectin. As shown in Fig. 8, antibody 16, like anti-4B4, was unique

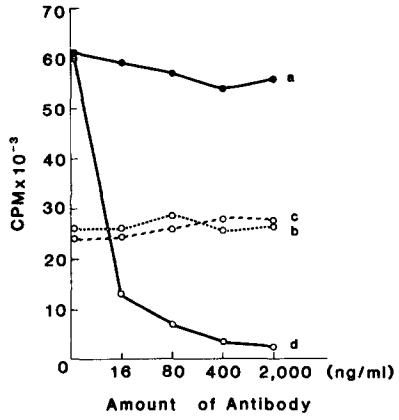

FIGURE 8. Antibody 16 inhibits the proliferation of CD4 cells induced by the combination of anti-CD3 antibody and fibronectin. (*a* and *d*) CD4 cells were cultured in anti-CD3 antibody and fibronectin-coated plates. (*b* and *c*) CD4 cells were cultured in anti-CD3 antibody-coated plates with IL2 (100 U/ml) or PMA (1 ng/ml), respectively. [^{3}H]Thymidine incorporation of CD4 cells stimulated by IL-2 or PMA alone was <1,000 cpm. Open circles represent the amounts of antibody 16 and closed circles represent the amounts of LFA 1 antibody used in this experiment. CD4 cells were cultured in serum-free medium in these plates for 4 d, after which proliferation was assessed by determining [^{3}H]thymidine incorporation. The data are expressed as the mean of triplicate samples. Each SE was <15%. When flat-bottomed plates were coated with fibronectin alone, <100 cpm of [^{3}H]thymidine incorporation was obtained. Antibody 16 antibody inhibits the proliferation of CD4 cells induced by anti-CD3 antibody and fibronectin but not the proliferation of CD4 cells induced by anti-CD3 antibody and IL-2 or PMA. The data are representative of three separate experiments.

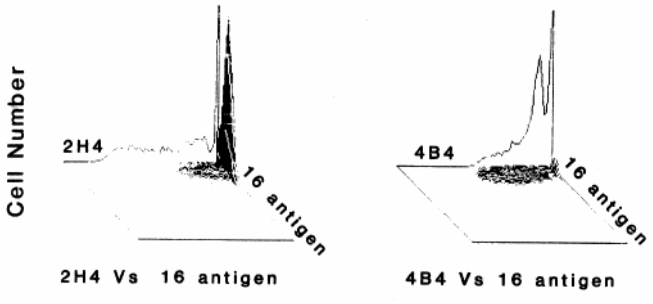

FIGURE 9. The relationship between antibody 16 antigen (VLA-5) and 2H4 and 4B4 antigens on CD4 cells. Two-color cytofluorographic analysis of CD4 cells with anti-4B4 antibody or anti-2H4 antibody conjugated to phycoerythrin, and antibody 16 (followed by FITC anti-rat Ig antibody) was performed on a logarithmic scale. Fluorescence intensities of 4B4, or 2H4, and 16 antigen are expressed on the *x*- and *y*-axis, respectively. The *y*-axis shows cell number. The data are representative of six separate experiments using CD4 cells from different healthy donors.

in its ability to inhibit the proliferation of CD4 cells in the presence of anti-CD3 antibody plus fibronectin. In contrast, the control antibody LFA-1 had no inhibitory activity. Moreover, anti-VLA-3 and anti-VLA-4 antibodies were also unable to inhibit anti-CD3 plus fibronectin-induced CD4 cell proliferation (data not shown). The above finding indicates that the VLA-5 protein acts as a fibronectin receptor on the CD4 lymphocytes and that this receptor-ligand interaction is of great importance to lymphocyte activation in this system. It should be noted that antibody 16 did not inhibit the activation of CD4 cells in the presence of anti-CD3 plus type I, III, or IV collagen, whereas anti-4B4 antibody could inhibit these responses (data not shown). However, as shown in Fig. 1, the proliferation of CD4 cells in the presence of anti-CD3 and type I, III, or IV collagen is significantly lower than that induced by anti-CD3 and fibronectin. Taken together, these results suggest that it is within the set of molecules recognized by anti-4B4 that is of key importance to lymphocyte activation in this system.

The Distribution of 16 Antigen (VLA-5) on Subsets of CD4 Cells. Next, the relationship between 16 antigen (VLA-5) and the 2H4 and 4B4 antigens on CD4 cells was determined by using double fluorescence staining. As shown in the representative experiment in Fig. 9, the majority of cells expressing VLA-5 antigen also expressed the 4B4 antigen (90%). In contrast, only 15% of the cells expressing the VLA-5 antigen coexpressed the 2H4 antigen. Furthermore, only low density 2H4 cells coexpressed the VLA-5 antigen. It should be noted that few, if any, CD4 cells expressed both 2H4 and VLA-5 at high antigen density. These studies further support the notion that the differential expression of the VLA-5 on distinct CD4 subsets may contribute to the previously defined functional differences between the $CD4^+4B4^+$ and $CD4^+2H4^+$ subsets (4, 7, 8).

Discussion

In the present study, we have demonstrated that fibronectin synergizes with anti-CD3 antibody in the activation of CD4 cells. Furthermore, our studies showed that the minimal fibronectin recognition sequence, RGDS (21, 27, 44), could block this

activation, and fragments of the fibronectin molecule containing the cell adhesive domain plus additional regions are required for this synergistic effect. The ability of fibronectin plus anti-CD3 antibody to induce CD4 cell activation could be blocked by anti-4B4 (CDW29), an anti-class I β integrin antibody. Moreover, we determined that anti-4B4 recognizes a set of VLA-5 heterodimers that can function as a fibronectin receptor on the CD4 lymphocytes. The fibronectin-VLA-5 interaction may be of substantial importance in lymphocyte activation.

In preliminary studies, we attempted to identify extracellular matrix protein receptors on CD4 lymphocytes by enriching for cells selectively bound to specific extracellular matrix proteins, including laminin, fibronectin, and collagen. However, using fibronectin-coated plates, we were only able to partially enrich for bound CD4 cells (data not shown). In the studies reported above, we developed a more sensitive assay system for the characterization of extracellular matrix protein receptors on CD4 cells. Since a number of matrix proteins are present in serum, and monocytes can produce fibronectin (45), our assay system relies on the use of highly purified CD4 cells in serum-free media. In this system, a mixture of anti-CD3 antibody and fibronectin could induce CD4 cellular activation, whereas neither could do it alone.

This synergistic effect of fibronectin with anti-CD3 antibody on CD4 proliferation appears to be specific since crosslinking of anti-CD3 antibody to other antibodies directed toward cell surface structures, such as CD4, class I, CD45R, and CD11a, did not induce proliferation in this system (data not shown). Second, enhanced proliferation can also be seen when soluble fibronectin is added to anti-CD3-coated plates that have been saturated with BSA to reduce nonspecific interactions between anti-CD3 and fibronectin (data not shown). These studies, as well as those presented above, suggested that binding of fibronectin to its receptor on CD4 cells may induce activation through the CD3-TCR complex. How this CD4 cell activation occurs is not entirely clear, but one piece of evidence presented above, that RGDS containing peptides and fibronectin fragments and anti-4B4, as well as anti-human fibronectin receptor (VLA-5) antibodies, block fibronectin-induced proliferation, suggests an important role for the fibronectin receptor in T cell activation.

A costimulatory effect of fibronectin on another type of cell activation has been observed in monocytes. Binding of fibronectin to its receptor on monocytes and costimulation with either C3b or IgG generates a signal that enhances phagocytosis of opsonized particles (46). Moreover, the 180-kD opsonic fibronectin fragment (180-kD opFnf), which has been purified from normal human plasma, augments monocyte phagocytosis of particulate activators of the human alternative complement pathway without affecting IgG- or C3b-mediated functions (34). The mechanism by which fibronectin enhances these cell functions is still not fully understood. It has been suggested that the interaction of fibronectin to its receptor might activate cells through the phosphorylation of a tyrosine residue on the VLA/integrin antigen family of β chain (47, 48), and other surface receptors containing phosphorylated tyrosine residues are known to be related to cell activation and cell growth (49).

In our studies, laminin had no effect on the proliferation of CD4 cells, whereas type I, III, and IV collagen had a modest effect compared with fibronectin (Fig. 1). Tomaselli et al. (50) reported that the receptor for laminin and type IV collagen on a rat neuronal cell line was likely to be VLA-1 and/or VLA-3 proteins, whereas the laminin receptor on platelets was reported to be VLA-6 (51). Others (14, 52)

have shown that VLA-2 can be a collagen receptor on leucocytes and other cells. In our studies, anti-4B4 (CDW29) antibody blocked the CD3-induced activation of CD4 cells with fibronectin as well as type I, III, or IV collagen. Antibody 16, in contrast, inhibited only CD4 cell activation when anti-CD3 was combined with fibronectin but had no effect in the presence of type I, III, or IV collagen. Results presented here strongly suggest that antibody 16 is directed toward the VLA-5 protein α subunit and that it only inhibited cell activation on anti-CD3 combined with fibronectin. Thus, fibronectin appears to interact with CD4 cells through the VLA-5 protein and synergizes with anti-CD3 antibody in the activation of CD4 cells. Antibody 16 recognizes a subset of the molecules reactive to anti-4B4. Thus, although anti-4B4 and antibody 16 appear to have similar distributions on CD4 and K562 cells, a small percentage of $4B4^+16^-$ and 16^+4B4^- cells do exist. In this regard, more recently Wayner et al. (53) have also described mAbs that specifically inhibit the adhesion of human cells to fibronectin, but not collagen or laminin. Moreover, the molecules recognized by these antibodies were shown to be identical to the fibronectin receptor and VLA-5.

The RGDS sequence contained within the fibronectin cell-adhesive domain appears to be involved in this synergistic activation of CD4 cells by anti-CD3. However, fragments containing the cell-adhesive domain alone were unable to induce the same degree of CD4 cell proliferation as that induced by intact fibronectin. This result suggests that additional regions of the fibronectin molecule outside of the cell-adhesive domain are also involved in the activation of CD4 cells. One possibility is that the other regions of the fibronectin molecule are required to stabilize the tertiary structure of the cell-adhesive domain, allowing its optimal binding to CD4 cells. Alternatively, these additional regions of the fibronectin molecule may themselves be required for maximal receptor-ligand interaction or crosslinking and subsequent cell activation. Further mutational analysis of fibronectin may help to clarify whether the presence of other fibronectin regions increases the efficiency of binding of the cell-adhesive domain to the fibronectin receptor or to the cell surface as a whole and how this is translated into an increase in CD4 cell activation detected by our culture system.

It has been suggested that fibronectin receptors have a role in anchorage and migration of various types of cells during maturation. Early lymphoid cells express the fibronectin receptor that may play a role in thymic colonization (30) and B cell differentiation (54). Moreover, it has been reported that the murine thymocytes that bind specifically to fibronectin are cortical thymocytes, suggesting that the fibronectin receptor on thymocytes is important in T cell differentiation (28). Similarly, erythroid differentiation is associated with a loss of cell adhesion to fibronectin, which correlates with a loss of fibronectin receptor expression (55). In addition to the positional roles of fibronectin receptors on various cells, our results indicate that the fibronectin receptor on CD4 lymphocytes with interaction of fibronectin may play some roles in lymphocyte activation and growth.

The fibronectin receptor (VLA-5) is also preferentially expressed on a subset of mature $CDW29(4B4)^+CD45R^-$ cells. The subset of CD4 mature cells responds maximally to soluble antigens in the presence of self MHC class II–bearing monocytes and provides good helper function for B cell Ig synthesis (7). The present results might explain the preferential response of $CD4^+CDw29^+$ cells to soluble antigens,

since the fibronectin receptor is involved in this unique function of this CD4 subset by augmenting activation through the CD3-TCR complex. In addition, it may explain the maximal signals for B cell help generated by this subset by augmenting cell interaction with B cells through fibronectin or other integrin family adhesive receptors. That monocytes produce fibronectin suggests that physiologic augmentation of antigen-specific T cell activation by fibronectin might occur when CD4 cells and monocytes interact, as well as in cases where antigens are presented in association with extracellular matrix proteins.

Summary

In this study, fibronectin synergized with anti-CD3 antibody to promote CD4 cell proliferation in a serum-free culture system. The cell-adhesive domain plus additional regions of the fibronectin molecule are involved in this synergy. Anti-4B4(CDw29) antibody blocked the activation of CD4 cells in this system. Furthermore, it is the VLA-5 protein within the set of molecules recognized by anti-4B4 that serves as a fibronectin receptor on the CD4 lymphocytes. The VLA-5 fibronectin receptor was mainly expressed on $CD4^+$ $CD45R^-CDw29^+$ cells and may in part contribute to the unique function of these cells.

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