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The Human 4F2 Antigen: Evidence for Cryptic and Noncryptic Epitopes and for a Role of 4F2 in Human T Lymphocyte Activation

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T lymphocyte activation can be triggered through multiple distinct, but functionally related, pathways. Murine monoclonal antibodies (mAbs) have been used to characterize the surface components of several of these pathways, as well as structures whose function is not yet known. One such cell surface structure is the heterodimeric 4F2 antigen, which is expressed on activated and proliferating cells. Two new mAbs that recognize the heavy chain of the 4F2 antigen have been produced in our laboratory. One antibody, UM7F8, is comitogenic with soluble anti-CD2 and immobilized (but not soluble) anti-CD3 mAbs. The second antibody, termed UM2G12, appears to recognize a cryptic epitope on the 4F2 heavy chain and is not comitogenic for T cells. In view of the functional effects seen with UM7F8, and the highly regulated expression of the 4F2 antigen, it seems likely that 4F2 has a specific role in T cell development and activation.

INTRODUCTION

The expression of several cell surface proteins can be induced or augmented when resting lymphocytes are stimulated to proliferate. Among these proteins is the human 4F2 antigen, which was originally detected by a murine monoclonal antibody (mAb) raised against the human T-cell tumor line HSB-2 (1, 2). This antigen is strongly expressed on all human tissue culture lines, the majority of malignant human cells, and peripheral blood (PB) monocytes (1, 2). While 4F2 is only expressed at low levels on resting T and B lymphocytes, a high level of expression can be readily induced following lectin or alloantigenic stimulation of resting T cells (2, 3). This antigen has also been detected in breast, esophageal, and colonic epithelium, keratinocytes (skin and hair follicles), renal proximal tubules, heart muscle, and embryonal but not adult fibroblasts (4–8).

The 4F2 antigen is a disulfide-linked 125-kDa heterodimeric membrane glycoprotein, composed of an 85-kDa glycosylated heavy chain and a 40-kDa nonglycosylated

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light chain (2). The epitopes recognized by both the original anti-4F2 antibody and subsequent anti-4F2 antibodies either reside on the heavy chain (1–5, 9–11) or have not been localized to heavy or light chain (6). The original antibody recognizes an epitope on the protein core (9).

A rat mAb (RL-388) raised against a variant of the EL4 thymoma cell line detects a similar murine 125-kDa surface disulfide-linked heterodimer and stimulates production of IL-2 (12). Peptide mapping demonstrated structural homology between the human and murine heavy chains and the light chains appeared to be identical (13). As no monoclonal antibodies have been produced which clearly recognize epitopes on the light chain, it is likely that this chain represents a protein of functional significance that has been highly conserved across species.

Two new monoclonal antibodies that recognize the 4F2 antigen have been produced in our laboratory and are described in this report. One antibody, termed UM7F8, is comitogenic with soluble anti-CD2 and immobilized (but not soluble) anti-CD3 mAbs, suggesting a functional relationship between 4F2 and both the CD2 and CD3 activation pathways. The second antibody, termed UM2G12, appears to recognize a cryptic epitope on the 4F2 molecule and lacks the functional effects seen with UM7F8. The epitopes recognized by both the original anti-4F2 antibody and these novel anti-4F2 antibodies reside on the heavy chain, as determined by flow cytometry of transfectants.

MATERIALS AND METHODS

Cells. Human peripheral blood mononuclear cells (PBMC) were isolated from venous blood by density gradient centrifugation using Histopaque (Sigma Chemical Co., St. Louis, MO). All blood donors were healthy volunteers. Human thymic tissue was obtained as excess surgical material from patients undergoing corrective pediatric cardiac surgery. The tissue was finely minced and passed through a wire mesh to obtain a single cell suspension. Mononuclear cells were then isolated using density gradient centrifugation using Histopaque. To activate human PBMC, freshly isolated cells (1×10^6/ml) were cultured in tissue culture flasks with the following stimuli: 0.5 μg/ml PHA (Murex Diagnostics, Atlanta, GA), 0.5 μg/ml ionomycin (Calbiochem, La Jolla, CA), 5 ng/ml PMA, or PMA + ionomycin. The human T leukemic cell line Jurkat was maintained in continuous cell culture in our laboratory. Murine 3T3 cells stably transfected with 4F2 heavy chain cDNA were the generous gift of Dr. Jeffrey Leiden (University of Chicago).

Antibodies. The monoclonal antibody designated UM7F8 (IgG1) was produced using a subtractive immunization technique to enhance the production of mAbs against rare or less immunodominant antigens (14). A 6-week-old female Balb/c mouse was serially immunized with cells of the human T-leukemic cell line Peer. Each immunization was followed by treatment with cyclophosphamide as previously described (14) (three immunizations at 2-week intervals). The same mouse was then immunized and boosted twice with cells from the thymocyte line Molt13. Hybridoma UM7F8 was subcloned and propagated in ascites form in pristane-primed mice. The mAb UM2G12 (IgM) was produced by conventional serial immunization with phorbol ester (PMA)-stimulated Jurkat cells. Other murine mAbs used include OKT3 (anti-CD3, IgG2a; American Type Culture Collection), 4F2 (anti-4F2, IgG2a; American Type Culture Collection), 1OLD24C1 (anti-$T11_2$, IgG2a; generous gift of Drs. E.

Reinherz and S. Schlossman), 1MONO2A6 (anti-$T11_3$; generous gift of Drs. E. Reinherz and S. Schlossman), 3C4E4 (anti-von Willebrand factor, IgG2a; produced in our laboratory), 3E2D10 (anti-von Willebrand factor, IgG1; produced in our laboratory), 1F12H2 (anti-von Willebrand factor, IgM; produced in our laboratory), and M1/42.3.9.8 (rat anti-mouse H-2, all haplotypes, IgG; American Type Culture Collection). FITC-conjugated goat anti-mouse IgG + IgM was purchased from Tago Inc. (Burlingame, CA), FITC-conjugated rabbit anti-rat IgG was purchased from Sigma Chemical Co., and FITC-conjugated mouse IgG was purchased from Coulter Immunology (Hialeah, FL).

Flow cytometry. Expression of cell surface antigens was assessed by direct or indirect immunofluorescence using an Epics C or Elite cell sorter (Coulter Electronics, Hialeah, FL). All antibodies were used at saturating concentrations. Positive cells were defined as those with fluorescence intensity above a threshold defined by the negative control (cells stained with NS-1, a nonspecific ascites fluid). Dead cells were excluded from analysis using forward- and right-angle light scatter gating.

Immunoprecipitation. Cells were surface labeled with ^{125}I by the lactoperoxidase method as described previously (15) and lysed in ice cold buffer containing 1% NP-40 and protease inhibitors (all reagents from Sigma Chemical Co.). The lysate was precleared by incubation with normal mouse serum (30 min at 4°C) followed by protein A–Sepharose beads (30 min at 4°C). The lysate was precleared a second time using an isotype-matched control mAb (anti-von Willebrand factor) and immunoprecipitated (1 hr at 4°C) using protein A–Sepharose beads covalently crosslinked to mAb UM7F8 or 4F2 by a method described previously (16). After extensive washing, the precipitates were analyzed by SDS–PAGE on 7.5% polyacrylamide gels, dried, and autoradiographed.

Proliferation assays. Human PBMC (1×10^5/well) were cultured in triplicate in 96-well round-bottom plates (Costar, Cambridge, MA) or 96-well sterile ELISA plates (Corning Inc., Corning, NY) in a final volume of 0.2 ml RPMI 1640 supplemented with 10% fetal calf serum (Life Technologies, Inc., Grand Island, NY), penicillin (100 U/ml)/streptomycin (100 μg/ml) (Irvine Scientific, Santa Ana, CA), and 2 m*M* glutamine (Life Technologies, Inc.). The ELISA plates were used for assays in which mAb OKT3 was immobilized onto the plates prior to the addition of cells or other stimuli. Costimuli included UM7F8, UM2G12, 4F2, 1OLD24C1 + 1MONO2A6, 1F12H2, 3C4E4, and 1 ng/ml PMA (Sigma Chemical Co.). Immobilized OKT3 was used at an initial concentration of 1 μg/ml with serial dilutions as indicated. Briefly, 96-well ELISA plates were incubated for 2 hr at room temperature with 10 μg/ml goat anti-mouse IgG in 50 m*M* Tris, pH 9.4 (Sigma Chemical Co.). After washing with phosphate-buffered saline, plates were incubated for 1 hr at room temperature with 1 mg/ml bovine serum albumin (Sigma Chemical Co.) and washed again. Plates were then incubated for 2 hr at room temperature with 1 μg/ml OKT3 or various dilutions as specified under Results. After washing, the plates were stored at −80°C until ready for use. Control wells were prepared in the same manner with the omission of OKT3. Proliferation was measured using [^{3}H]thymidine incorporation. The cultures were pulsed on Day 4 with 0.8 mCi/well [^{3}H]thymidine (New England Nuclear, Boston, MA), harvested 18 hr later (PHD cell harvester; Cambridge Technology, Inc., Watertown, MA), and counted in a liquid scintillation counter (Tri Carb 1500; Packard Instrument Co., Downers Grove, IL).

RESULTS

Two novel monoclonal antibodies recognize the 4F2 antigen. The monoclonal antibody designated UM7F8 (IgG1) was produced using a subtractive immunization technique as described above. The hybridoma designated UM7F8 produced an antibody that reacted with all human thymocytes, T cell clones, and T cell lines at strong fluorescence intensity by flow cytometry (FACS), but showed only weak staining of resting human PB T cells. Immunoprecipitation studies, as shown in Fig. 1, demonstrate that the UM7F8 antibody recognizes the 4F2 antigen. Thus, under reducing conditions, UM7F8 immunoprecipitated a heterodimer identical to that immunoprecipitated with the anti-4F2 antibody (lanes 1–6), and a single identical band was obtained with each antibody under nonreducing conditions (lanes 7–9). Preclearing with the anti-4F2 antibody totally removed the specific bands precipitated by either anti-4F2 or UM7F8 antibodies (lanes 4–6 and 10–12). The better visualization of the light chain in the immunoprecipitation experiment reflects more efficient external labeling of the light chain and does not indicate that either of the mAbs react with an epitope on the light chain. The band at approximately 45 kDa in lanes 2 and 3 probably represents actin. Two-dimensional gel electrophoresis of 4F2 and 7F8 immunoprecipitates confirmed coprecipitation of actin (data not shown).

The expression of the epitope recognized by mAb UM7F8 on various cell types was analyzed by flow cytometry. This epitope on the 4F2 antigen is expressed on 85% of resting peripheral blood T cells at a low density, whereas >95% of thymocytes and Jurkat cells express the antigen at higher density (Fig. 2).

The mAb UM2G12 (IgM) was produced by conventional serial immunization with phorbol ester (PMA)-stimulated Jurkat cells. Using FACS analysis, UM2G12 and UM7F8 were shown to crossblock each other, as well as the original 4F2 antibody. Both of these new antibodies recognize the 4F2 heavy chain, as determined by reactivity

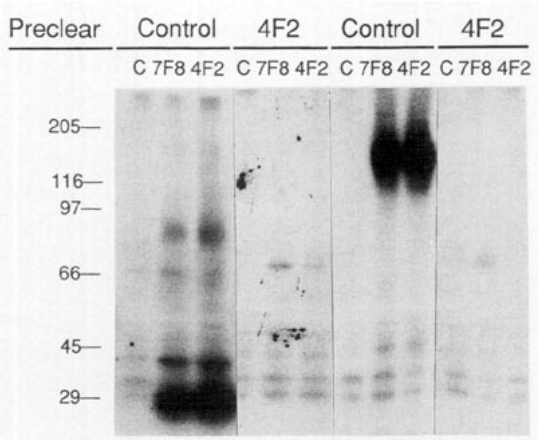


FIG. 1. Immunoprecipitation of the 4F2 antigen from lysates of ^{125}I-labeled Jurkat cells. Lanes 1–3, lysate precleared with control mAb and precipitated with the designated mAb (run under reducing conditions); lanes 3–6, lysate precleared with mAb 4F2 and precipitated with the designated mAb (reducing conditions); lanes 7–9, lysate precleared with control mAb and precipitated with the designated mAb (nonreducing conditions); lanes 10–12, lysate precleared with mAb 4F2 and precipitated with the designated mAb (nonreducing conditions).

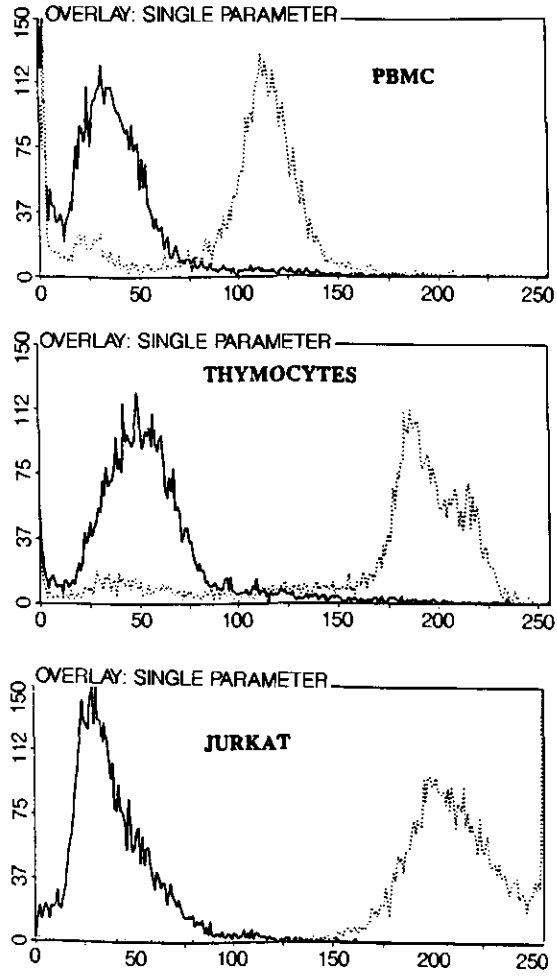


FIG. 2. Flow cytometric analysis of human peripheral blood lymphocytes, thymocytes, and Jurkat cells stained with UM7F8 or control ascites.

with murine fibroblasts (NIH 3T3 cell line) stably transfected with human 4F2 heavy chain cDNA (Fig. 3). UM2G12 was not a satisfactory reagent for immunoprecipitation.

The epitope recognized by UM2G12 does not appear to be identical to those recognized by either the original 4F2 antibody or UM7F8. The UM2G12 epitope is barely detected above background fluorescence levels on resting PBMC (Figs. 4 and 5) or E^+ (data not shown) in contrast to the reproducible, albeit low-intensity, detection of the 4F2 and UM7F8 epitopes. The UM2G12 epitope is expressed on T cells following activation, but at a lower density than the epitope(s) recognized by the other mAbs (Fig. 5). This difference is demonstrated at saturating antibody concentrations and does not reflect the difference in isotypes (IgG versus IgM) (data not shown). Thus, UM2G12 appears to recognize a cryptic epitope on the 4F2 heavy chain, analogous to the $T11_3$ epitope on the CD2 molecule.

We have also shown, by FACS analysis, that all three mAbs (UM7F8, UM2G12, and 4F2) react with portions of the molecule that are resistant to destruction by neur-

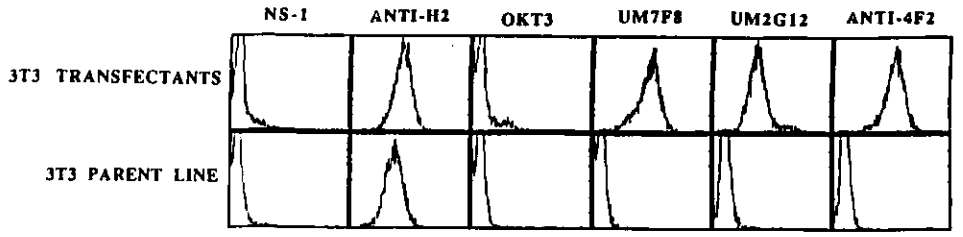


FIG. 3. Cell surface staining of 4F2 heavy chain transfectants. Murine fibroblasts stably transfected with human 4F2 heavy chain cDNA were stained with NS-1 (negative control), anti-H2 (positive control), OKT3, UM7F8, UM2G12, and mAb 4F2. Cells from the untransfected parent line were similarly treated.

aminidase, trypsin, pronase, and staphylococcal V8 protease, upon treatment of intact cells with concentrations of these enzymes that permit retention of cell viability (data not shown).

Functional effects of anti-4F2 monoclonal antibodies. The functional effects of these novel mAbs are distinct. The UM7F8 mAb is comitogenic for T cells in the presence of suboptimal concentrations of soluble anti-CD2 antibodies (anti-$T11_2$ + anti-$T11_3$) or immobilized (but not soluble) anti-CD3 (OKT3) antibodies (Fig. 6). In multiple experiments, significant synergy between CD2 and 4F2 was observed in about 50% of experiments and between 4F2 and CD3 in most experiments. To a lesser extent, similar effects are observed using the original 4F2 mAb (data not shown). Using the UM2G12 mAb, however, no such effects are observed. Thymocytes express 4F2 at moderate density but do not proliferate in response to a combination of mAb UM7F8 and either anti-CD2 or anti-CD3 mAbs. Several cloned $CD4^+$ or $CD8^+$ T cell lines have also failed to show augmented proliferation in the presence of UM7F8 (data not shown).

We have used flow cytometry to examine the effects of mAbs UM7F8 and UM2G12 on calcium ion fluxes in Jurkat cells loaded with the dye Indo-1. Neither UM7F8 nor UM2G12 is able to induce a calcium ion flux on its own or in conjunction with a suboptimal concentration of anti-CD2 or anti-CD3 mAbs (data not shown).

DISCUSSION

The function of the 4F2 antigen has not yet been fully established, although several interesting observations have been made. Since the antigen is expressed primarily on the surface of activated cells, studies to date have attempted to describe the mechanism by which the antigen affects cellular activation.

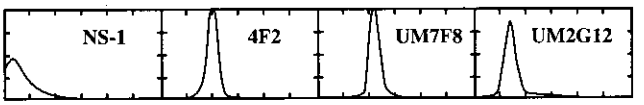


FIG. 4. Cell surface staining of resting human PBMC with anti-4F2 monoclonal antibodies. Resting PBMC were stained with NS-1 (negative control), mAb 4F2, UM7F8, and UM2G12. Mean channel fluorescence was 3.21 for anti-4F2, 4.96 for UM7F8, and 1.07 for UM2G12. The histograms are displayed on a three log scale.

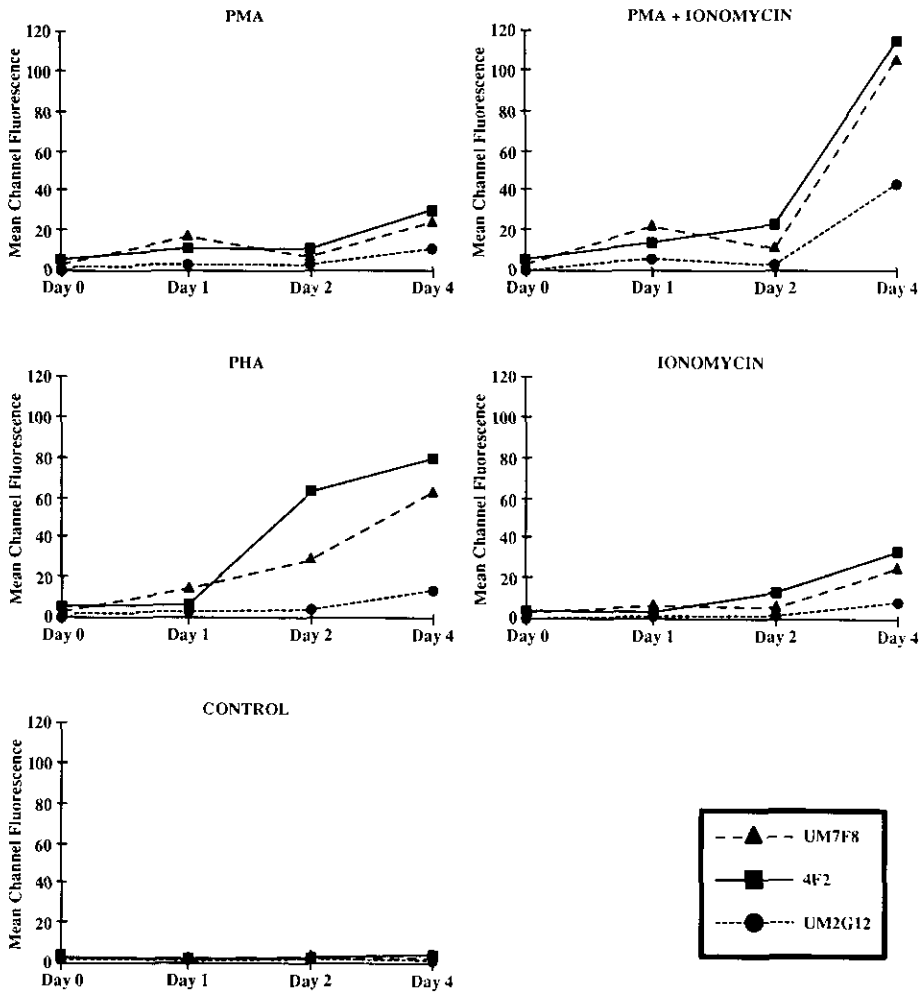
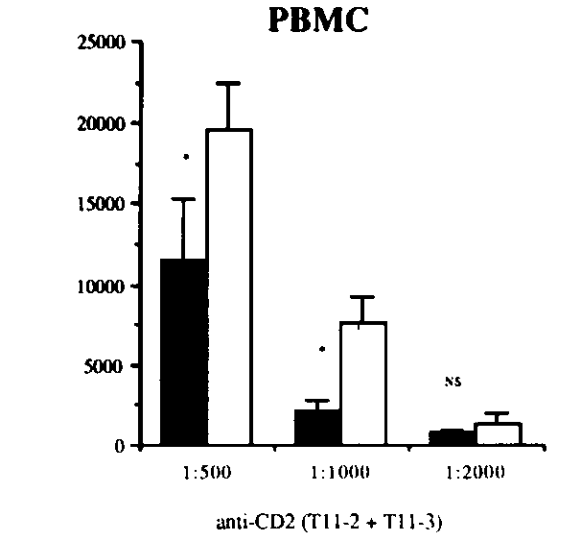


FIG. 5. Effect of activating stimuli on the surface expression of 4F2 heavy chain epitopes. Resting human PBMC were stimulated for up to 4 days with PMA, ionomycin, PMA + ionomycin, or PHA (as described under Materials and Methods). Culture medium without activating stimuli was used as control. After washing, cells were stained with UM7F8, mAb 4F2, and UM2G12 and analyzed by flow cytometry. The mean channel fluorescence is expressed on a linear scale. Density of the 4F2 and UM7F8 epitopes was typically fourfold the density of the UM2G12 epitope. This difference is most easily visualized in Fig. 5 for the fully activated cell populations. Similar findings were observed in additional experiments with activated T cells and with flow cytometric analysis of a panel of transformed cell lines.

Some evidence suggests that 4F2 is involved in the regulation of intracellular Ca^{2+} concentration. Incubation of cultured human parathyroid adenoma cells with anti-4F2 raises the concentration of intracellular Ca^{2+} and results in a decrease in the basal level of PTH secretion by these cells (18). Preincubation of skeletal muscle or cardiac sarcolemmal vesicles with anti-4F2 inhibits 90% of the Na^{+}-dependent Ca^{2+} uptake by these vesicles (4).

The 4F2 antigen has been proposed as a target structure for natural killer (NK) cells. Early studies demonstrated that anti-4F2 did not inhibit NK cell activity (2),


PBMC
25000
20000
15000
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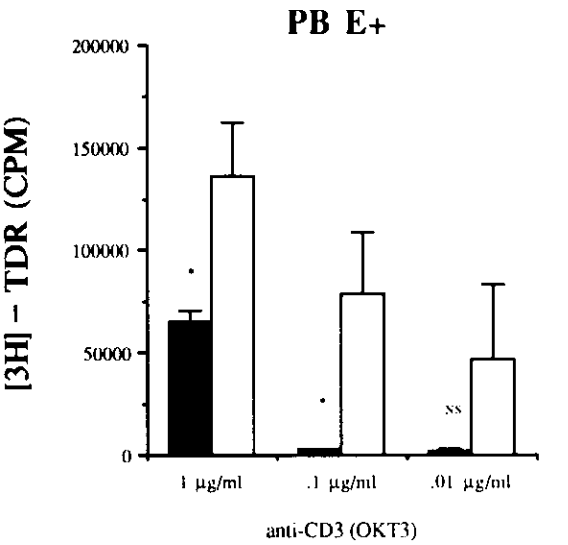
ns
1:500
1:1000
1:2000
anti-CD2 (T11-2 + T11-3)


PB E+
[3H] – TDR (CPM)
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1 µg/ml
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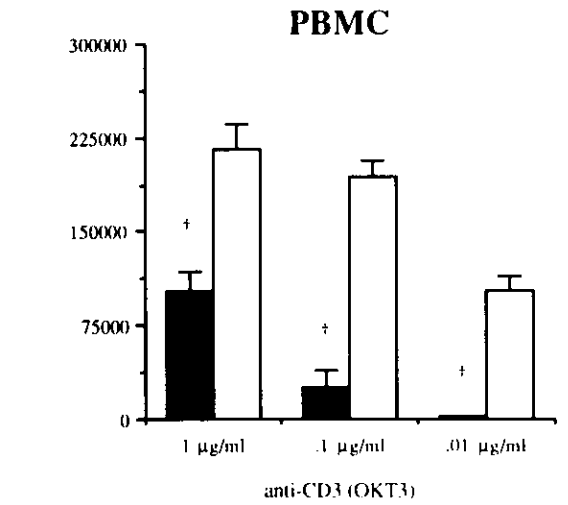
PBMC
300000
225000
150000
75000
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.01 µg/ml
anti-CD3 (OKT3)

nor does the CB43 mAb (6, 17). However, a human cloned NK cell line was developed which specifically recognized and killed target cells bearing the 4F2 antigen on their surface (11). This correlates with the observation that NK or "NK-like" activity is directed primarily against activated and proliferating cells.

Further evidence that the 4F2 antigen is involved in T cell activation comes from the observation that augmented expression is observed within 4–6 hr of lectin stimulation (3, 17), earlier than the appearance of either the transferrin or IL-2 receptor molecules, or the onset of DNA synthesis.

As mentioned previously, 4F2 is expressed on most human tumor cells. Murine mAbs have been raised against both rat and human bladder cancer cell lines, which recognize the 4F2 antigen and its rat homologue. Addition of these antibodies to the corresponding tumor cell lines inhibited DNA synthesis and proliferation of the tumor cells *in vitro* in a dose-dependent manner. The cells regrew when freed from antibody, which did not cause down-regulation of the cell surface antigen (10). These observations again suggest that the 4F2 antigen (and its rodent homologue) plays an important role in cellular proliferation.

Injection of *in vitro* transcribed 4F2 heavy chain RNA has recently been found to stimulate the uptake of dibasic and neutral amino acids into *Xenopus* oocytes (19, 20). The investigators suggested that 4F2 may function as an activator of a single broad-spectrum native oocyte transporter, possibly a sodium cotransporter. The function of the 4F2 light chain was not addressed in these reports, and to date, its function remains entirely unknown.

Though 4F2 has generally been thought of as an antigen expressed on activated but not resting T cells, staining with mAb UM7F8 demonstrates that this epitope on 4F2 is expressed on 80–90% of resting human PBMC at a low density. In contrast, greater than 95% of postnatal human thymocytes express the antigen at significantly higher density, comparable to that of the leukemic T cell line Jurkat. Of note, the majority of these thymocytes do not normally express activation markers such as IL-2 receptors, nor are they actively proliferating. 4F2 expression is therefore highly regulated during T cell development. All thymocytes, including immature double-positive ($CD4^+CD8^+$) and mature single-positive ($CD4^+$ or $CD8^+$) cells express 4F2 at high density, whereas resting peripheral T cells express it at very low density and then reacquire high-density surface expression following activation. This suggests an important role for this structure in T cell function, possibly with distinct roles at different developmental stages.

Both of the new mAbs described above crossblock the original 4F2 antibody, as demonstrated by flow cytometry. Despite this observation, it is likely that the epitope recognized by mAb UM2G12 is distinct from those recognized by either mAb 4F2 or UM7F8. UM2G12 appears to recognize a cryptic 4F2 epitope which is exposed and/or up-regulated after resting T cells are activated, analogous to the $T11_3$ epitope on CD2. In addition, the cell surface density of this epitope never reaches that of the epitopes recognized by mAb 4F2 or UM7F8, even after T cell activation.

The functional effects of mAb UM7F8 differ from those obtained with previous anti-4F2 antibodies. The original anti-4F2 antibody was not reported to be mito-

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FIG. 6. Comitogenic effects of mAb UM7F8. Human PBMC or PB E-rosette-positive cells were cultured with suboptimal concentrations of anti-CD2 mAbs ($T11_2$ + $T11_3$) or anti-CD3 (OKT3), in conjunction with either UM7F8 or an isotype-matched control Ab. The results are expressed as the mean net cpm of triplicate cultures ± SD. $^*P < 0.05$, $\dagger P < 0.005$. NS, difference not significant.

genic for PBMC and did not block T cell responses to allogenic cells. 4F2 antibody did, however, inhibit mitogen (concanavalin A)-induced proliferation of PBMC by 50% (2).

Recently, a mAb termed CB43 (IgM isotype) was produced which has been proposed to recognize an epitope on the light chain or one dependent upon the conformation of the molecule (6). The cDNA for the 4F2 heavy chain (see below) was transfected into murine fibroblasts and was undetectable using CB43. The possibility that the epitope is a carbohydrate moiety on the heavy chain which might not be expressed on transfected murine cells was not excluded. In one study, the CB43 antibody inhibited the mitogenic (PHA) response of T cells by 40%, without affecting the response to allogenic cells (6). In another, no inhibition of the PHA response was noted and CD43 was able to induce proliferation of PBMC in 4/16 donors, although the stimulation index never exceeded 3–4. Proliferation of T lymphoblasts and several established cell lines was not affected. In that study, CB43 was comitogenic with soluble anti-CD3 mAb, and production of IL-1β, but not IL-2 or IFN-γ, was noted. Finally, PHA-induced proliferation of T cells was enhanced in the presence of CB43-pretreated monocytes (17).

The UM7F8 mAb is comitogenic for T cells in the presence of suboptimal concentrations of soluble anti-CD2 mAbs and immobilized anti-CD3 mAb, suggesting a functional relationship between 4F2 and both of these activation pathways. The comitogenic effect of UM7F8 on PB E^+ lymphocytes stimulated with immobilized anti-CD3 suggests that macrophages may not be required for this functional effect, but an accessory cell requirement has not yet been entirely excluded.

The role of the 4F2 antigen in T cell signaling is unknown. The early calcium ion flux is not augmented by UM7F8, UM2G12, or mAb 4F2. It is not yet known if 4F2 is a phosphoprotein (either constitutively or after activation), or if costimulation with UM7F8 and anti-CD2 or anti-CD3 affects the phosphorylation of other T cell proteins.

Studies of the 4F2 antigen have been limited by the lack of monoclonal antibodies which specifically recognize epitopes on the light chain. As the light chain appears to be more highly conserved across species than the heavy chain, many of the functionally important domains of 4F2 may reside on the light chain, which has yet to be cloned.

Thus, the 4F2 antigen is likely to play an important functional role in human T cell activation and proliferation. This role may be restricted to lymphocytes, or common to nonlymphoid systems as well, based on the high surface density of 4F2 on virtually all rapidly proliferating cells.

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Note added in proof. The 4F2 antigen has been designated CD98 by the Fifth Human Leukocyte Differentiation Antigen Workshop.

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