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ARTICLES

Triggering of the Proteinase Dipeptidyl Peptidase IV (CD26) Amplifies Human T Lymphocyte Proliferation

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Abstract CD26 (Ta1, dipeptidyl peptidase IV) is a M_r 105,000 protein expressed at high levels on activated T lymphocytes and is a potential marker of memory T cells. Reciprocal immunodepletion and solid phase double determinant binding studies showed that mAb AC7 and the CD26-specific mAb anti-Ta1 reacted with spatially distinct sites on the same molecule. The proteinase dipeptidyl peptidase IV (DPP IV) was immunoprecipitated with mAb AC7 and its enzymatic activity directly assayed using an enzyme overlay membrane system. High levels of DPP IV activity were detected on the T cell tumor line CCRF-HSB-2 and on PBMC stimulated by a variety of methods. By itself, soluble mAb AC7 was not mitogenic for T cells but enhanced T cell proliferation that resulted from treatment with phorbol myristic acetate (PMA) in the presence of accessory cells. T cell proliferation was also induced by co-immobilized mAb AC7 and mAb OKT3 (anti-CD3). Cultures of T cells growing in the presence of IL-2 responded with accelerated growth when exposed to a combination of immobilized mAb AC7 and soluble mAb OKT3, a result not seen with freshly isolated T cells.

Key words: T cell proliferation, enzyme overlay membrane, T cell proteinase, Ta1, memory

During the life span of a T lymphocyte a series of phenotypic alterations occur. An initial set of changes arises in the thymus where thymocytes differentiate into discrete T cell subclasses [1,2]. Two of the best characterized subclasses are the major histocompatibility complex (MHC) class I-specific $CD8^+$ cells and the MHC class II-specific $CD4^+$ cells [3–5]. Other, sometimes overlapping, subsets are characterized by the cell surface antigen CD29 [6,7] and the CD45 markers defined by the mAb 2H4 (CD45RA) and UCHL1 (CD45RO) [6–8]. Functional T cell subsets include helper (and/or inducer) cells, suppressor cells, and cytotoxic T lymphocytes [9].

Further changes in cell surface phenotype occur during the activation of resting T lymphocytes by antigens or lymphokines [10]. A partial list of those T cell activation associated changes include the appearance of, or increase in, the IL-2 receptor [11], the integrins VLA-1, VLA-2, and VLA-4 [12,13], Pgp-1 [14], insulin and transferrin receptors [15,16], and the Ta1 antigen [17,18] as well as alterations in the ratios of the CD45RA and CD45RO epitopes [7,19].

The Ta1 molecule is a M_r 105,000 protein expressed on a subset of peripheral blood T lymphocytes that are believed to contain the memory T cell population [17,18,20]. Activation with antigen, mitogen, or mAb anti-CD3 causes a dramatic increase in the number of $Ta1^+$ lymphocytes and in the level of Ta1 expression [18,19]. Also, the number of T cells expressing Ta1 is increased in auto-immune diseases such as multiple sclerosis, Graves disease, and retinitis pigmentosa [21–23].

The enzyme dipeptidyl peptidase IV (DPP IV; E.A.C. 3.4.14.5) is a cell surface proteinase expressed on a range of different cell types, including liver, pancreas, and placenta [24,25]. DPP IV is also expressed, at very low levels, on a subset of resting T lymphocytes [26]. The DPP IV^+ lymphocytes account for almost all IL-2 secretion [27] and are important in initiating immunoglobulin synthesis by B lymphocytes [28]. Evidence has been presented that the CD26 antigen of T lymphocytes is actually DPP IV

Abbreviations used: DPP IV, dipeptidyl peptidase IV; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cells; VLA, very late (activation) antigens.

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[29,30], and it has recently been suggested that DPP IV is capable of binding to fibronectin and collagen [31–33]. In the present study we describe the specificity of mAb AC7 for the CD26 antigen and the ability of CD26-specific mAbs to promote T cell proliferation.

MATERIALS AND METHODS

Cell Lines

Human T cell tumor and B lymphoblastoid cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. The long term $CD8^+$, HLA-A2 allospecific human cytolytic T lymphocyte line, A α JY, was generated as previously described [34] and provided by Dr. Alan Krensky, Stanford University School of Medicine, Stanford, CA. Non-specifically activated T cells were either produced in this laboratory or were the generous gifts of William L. Crump III, M.D. Anderson Cancer Center, Houston, TX. Activation protocols included treatment of peripheral blood mononuclear cells (PBMC) with 200 U/ml IL-2 every four days, 10 ug/ml phytohemagglutinin (PHA), and an initial treatment of PBMC with 10 ng/ml of mAb OKT3 in serum free RPMI-1640 medium followed after 48 hours by culture in medium supplemented with 10% FBS and 250 units/ml IL-2.

Antibodies

Monoclonal antibodies were used as ascites, or purified from ascites fluid by a two step procedure (see below). The mAb anti-Ta1 [17] was provided by Dr. Alan Krensky, Stanford University School of Medicine, Stanford, CA. The mAbs OKT3 (anti-CD3), OKT4 (anti-CD4), AC7, D2, and the anti-HPB-ALL TCR_{id} mAb T40/25 [35] were maintained in this laboratory. Ascites of the VLA-4-specific mAb L25 was the generous gift of Dr. Elizabeth Evans (Becton Dickinson, Mt. View, CA). The mAb AC7 is an IgG2b antibody made by fusing SP2/0 myeloma cells with draining lymph node lymphocytes of a BALB/c mouse immunized with immuno-purified activation antigens [36]. The mAb D2 is an IgG2b antibody of unknown specificity used as an isotype control for mAb AC7. To purify mAb, ascites fluid proteins were precipitated with 50% ammonium sulfate dissolved in 40 mM sodium phosphate, pH 8.0, and the IgG fraction isolated by DEAE-cellulose chromatography (DE-52, Whatman, Hillsboro, OR).

Radiolabeling

Purified mAb were radiolabeled using 1,3,4,6-tetrachloro-3a,6a,diphenylglycoluril (IODOGEN, Pierce Chemical Co., Rockford, IL) as previously described [37]. Cell surface proteins were labeled by a modification [38] of the lactoperoxidase-catalyzed radioiodination procedure of Keski-Oja et al. [39]. Radioiodinated cells were lysed in 10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide containing 0.5% Nonidet P-40 (NP-40). After 30 min lysis cellular debris was removed by centrifugation at 18,000*g* for 20 minutes.

Immunoprecipitation

Cell lysates were precleared for 60 min at 4°C with 10% (v/v) formalin-fixed Staphylococcus aureus Cowan I (SACI, Calbiochem, La Jolla, CA) at 2.5×10^6 cell equivalents/50 ul SACI. The pre-cleared supernatant was collected after centrifugation at 5000*g* for 2 min to pellet the SACI. Immunoprecipitations were done in the following manner: 50 ul of 10% SACI was incubated with 2.5 ul of affinity purified rabbit anti-mouse Ig (Cappel/Organon Teknika Corp., West Chester, PA) for 1–2 hrs at 4°C, then washed three times with lysis buffer to remove unbound antibody. The resulting RAMIg-SACI was incubated with 5–7 ul of ascites fluid for 2–4 hrs at 4°C to immobilize the mAb. The resulting complexes (mAb-RAMIg-SACI) were washed three times with lysis buffer, then added to the precleared cell lysates. After incubation for 4–6 h at 4°C, the immune complexes were washed three times with lysis buffer, and bound antigen was eluted into SDS-PAGE sample buffer.

Electrophoresis

One-dimensional SDS-PAGE was carried out on vertical 7.5% gels according to the method of Laemmli [40]. Gels containing radioactive samples were dried and autoradiographed using intensifying screens (Cronex Lightning Plus, DuPont, Boston, MA) at −80°C with Kodak XR film.

Solid Phase Double Determinant Assay

Purified antibody (20 ug/ml) in PBS was added to the wells of a polyvinyl chloride microtiter plate (50 µl/well) and incubated for 25 hr at 4°. Excess antibody was aspirated and the plates washed three times with PBS. Each well was then treated with 250 ul of 0.5% bovine serum

albumin (BSA) in PBS for 4 hr at room temperature to block any free protein binding sites and washed three times with PBS. Unlabeled HPB-ALL or A α JY cells were lysed at 5×10^7 cells/ml in NP-40 lysis buffer for 30 minutes and the cellular debris removed by centrifugation. Lysates were diluted with an equal volume of 0.5% BSA/PBS, and 50 ul of this mixture was added to each well. After incubation for 5 hr at 4°C, the wells were washed 4 times with lysis buffer diluted with an equal volume of 0.1% BSA/PBS (wash buffer). 1×10^6 cpm of radioiodinated antibody in 50 ul of wash buffer was added to each well and incubated for 1 hr at 4°C. The plates were washed five times with wash buffer to remove unbound antibody, and the wells counted for bound radioactivity.

Analysis of DPP IV Activity

Dipeptidyl peptidase activity was detected using an Enzyme Overlay Membrane (EOM, Enzyme Systems Products, Dublin, CA) to which the substrate ALA-PRO-AFC (7-amino-4-trifluoromethyl Coumarin) had been coupled [31,41,42]. Cell lysates or immune complexes were incubated at room temperature in sample buffer (10% glycerol, .003% bromophenol blue in 58 mM Tris/HCl, pH 6.8 with or without 2% 2-mercaptoethanol) to retain DPP IV activity [25,31] and resolved by SDS-PAGE. The EOM was moistened in 0.2–0.5 M Tris/HCl pH 7.8, placed against the gel, and incubated at 37°C for 15 min in a humidified atmosphere. The membrane was then removed from the gel and placed atop a long-wavelength ultraviolet lamp box. Removal of the dipeptide ALA-PRO from the fluorogenic AFC results in the appearance of fluorescent bands on the membrane [31,41,42] which were then photographed.

Isolation of Peripheral Blood Cells and Proliferation Assays

PBMC were isolated by centrifugation on a cushion of Lymphocyte Separation Medium (LSM, Organon Teknika, Durham, NC) and resuspended in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine. T cells were isolated from the PBMC by a one hour incubation on plastic followed by treatment with Myoclear™ (Beckman Instruments, Fullerton, CA) to remove the monocytes and a T Cell Column™ (Beckman) to remove B cells. Both Myoclear™ and the T Cell Column™ were used according to suppliers' instructions. The assay conditions consisted of 50,000 cells/well (triplicate cultures for each data point) in 96-well tissue culture plates in a total volume of 300 ul/well. After 3–5 days of culture, plates were pulsed for 24 hr with 1.0 uCi/ml tritiated thymidine. Samples were harvested using a PhD cell harvester (Cambridge Technology Inc, Cambridge, MA) and assayed for the incorporation of radioactivity by liquid scintillation counting. PBMC proliferation assays utilized soluble antibody either as ascites, or as a DE52 IgG fraction. Experiments with purified T cells were done using soluble (ascites) or immobilized (IgG) antibody. Antibodies were covalently immobilized to plastic using sterile 96-well CovaLink (Nunc Inc., Naperville, IL) plates according to the manufacturer's instructions. Briefly, the mAb was diluted in PBS to the appropriate concentration, 30 ul added to each well and incubated at room temperature. Excess mAb was removed by aspiration, the wells washed three times with 5 mg/ml BSA (in PBS), and 125 ul of BSA/PBS was added and incubation continued for a further 2 hr at room temperature. Plates were stored at 4°C until use. The IgG fraction of mAb OKT3 was immobilized at a concentration of 100 ng protein/well and incubated for 3 hr and mAb AC7 immobilized at 500 ng/well with 2.5 hr incubation. In wells containing more than one mAb the addition of IgG was stepwise (i.e., 30 minutes OKT3, the addition of AC7 and 2.5 hr of incubation). Radioimmunoassay indicated that the CovaLink plates could bind approximately 40–50 ng/well of mAb OKT3 and 50–75 ng/well mAb AC7 under the incubation conditions using two antibodies. In wells containing a single mAb approximately 50 ng/well mAb OKT3 or 200 ng/well mAb AC7 bound.

RESULTS

The mAb AC7 was derived using a protocol designed to optimize the production of antibodies specific for T lymphocyte activation molecules. A rabbit antiserum was produced by immunization with a long term IL-2 dependent $CD8^+$ CTL line followed by extensive absorption with the $CD2^+$, $CD3^+$, $CD4^+$, $CD8^+$ T cell tumor line HPB-ALL. The absorbed antiserum was used to make immune complexes which were used for hybridoma immunization [36].

mAb AC7 Reacts With an Epitope of the T Cell Activation Antigen Ta1

The expression of the mAb AC7-reactive antigen on resting and activated peripheral blood

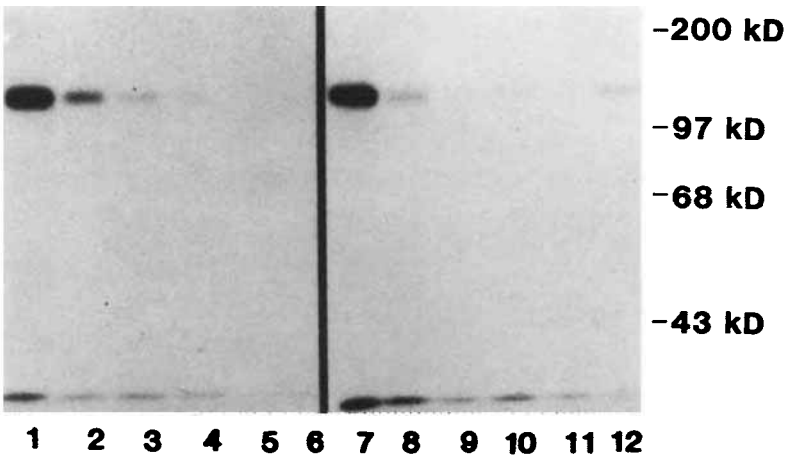

Fig. 1. Immunodepletion analysis of T cell surface antigens reactive with monoclonal antibodies anti-Ta1 and AC7. In the left panel, 50 μl NP-40 lysate of a $CD8^+$ CTL (A α JY) was subjected to five sequential immunoprecipitations with mAb anti-Ta1 (**lanes 1–5**) and followed by immunoprecipitation with mAb AC7 (**lane 6**). In the right panel, lysate was immunoprecipitated with mAb AC7 (**lanes 7–11**) and followed by mAb anti-Ta1 (**lane 12**). The precipitates were heated at 100°C for 5 minutes, then analyzed by SDS-PAGE under reducing conditions.

mononuclear cells (PBMC) was characterized by FACS analysis and immunoprecipitation. Freshly isolated PBMC contained very few (< 5%) $AC7^+$ cells with very low levels of AC7 expression. After treatment of the cells for one week with anti-CD3 + 250 U/ml IL-2, the number of $AC7^+$ cells rose to over 80% and the level of mAb AC7 binding per cell increased over fiftyfold. The AC7 mAb immunoprecipitated a single polypeptide of M_r 105,000 from PBMC activated by four different protocols including IL-2, anti-CD3, PHA, or alloantigen. Freshly isolated PBMC did not contain detectable amounts of mAb AC7-reactive material as assayed by radioimmunoprecipitation. These experiments indicated that mAb AC7 reacted with a molecule that is upregulated during lymphocyte activation and proliferation (data not shown).

A T cell activation marker of M_r 105,000 is the CD26 antigen [17,18]. To determine if mAb AC7 reacted with the CD26 molecule, we performed serial immunodepletions using both mAb AC7 and the CD26 specific mAb anti-Ta1. The IL-2 dependent $CD8^+$ anti-HLA A2 specific CTL line A α JY was surface radioiodinated and mAb anti-Ta1 reactive material removed by sequential immunoprecipitation (Fig. 1, lanes 1–5). Depletion by mAb anti-Ta1 removed all of the mAb AC7 reactive material (Fig. 1, lane 6). In the reciprocal experiment, depletion of anti-Ta1 reactive material by mAb AC7 was clearly seen but was slightly less efficient (Fig. 1, lanes 7–12).

The reactivity of mAb anti-Ta1 and AC7 was also compared in a solid phase double determinant radioimmunoassay. Antigen captured by mAb anti-Ta1 was bound by ^{125}I-labeled mAb AC7 (Fig. 2). In the reciprocal experiment, mAb AC7 captured material was reactive with ^{125}I-labeled mAb anti-Ta1. These results confirm that both mAb react with the same molecule. Furthermore, antigen captured by immobilized mAb AC7 was not bound by soluble ^{125}I-labeled mAb AC7 indicating the existence of a single mAb AC7 binding epitope per molecule. The same result was seen for mAb anti-Ta1 (Fig. 2). Since the binding of mAb AC7 and anti-Ta1 was noncompetitive and there were single epitopes reactive with each antibody, we concluded that mAb anti-Ta1 and mAb AC7 react with spatially distinct sites on the same molecule.

mAb AC7 Reacts With the Proteinase Dipeptidyl Peptidase IV

The anti-CD26 mAb CB.1 (anti-Tp103) has been used to co-purify DPP IV enzymatic activity [30]. To determine if mAb AC7 also co-purified dipeptidase activity we used an Enzyme Overlay Membrane (EOM) coupled with the DPP IV–specific substrate ALA-PRO-AFC [31,41,42] and employed the ability of DPP IV to retain enzymatic activity after reduction and resolution on polyacrylamide gels containing sodium dodecyl sulfate (SDS) [25]. The sequence X-PRO-X, where proline is the penultimate amino terminal amino acid, is the primary substrate for DPP IV [24,25]. Schön et al. have shown that the only lymphocyte enzyme capable of cleaving this substrate is DPP IV [43], and Piazza et al. have utilized EOM to identify rat

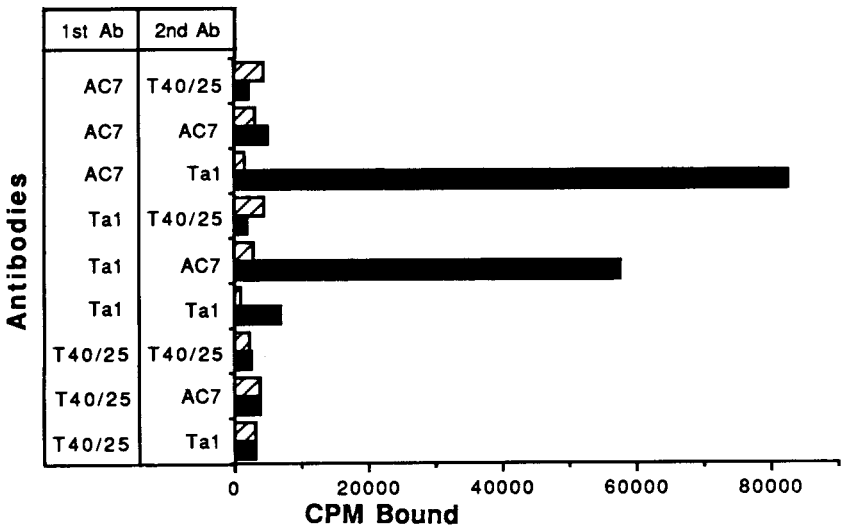

Fig. 2. The binding of mAb anti-Ta1 and AC7 is noncompetitive. The anti-CD26 reactive mAb AC7 and anti-Ta1 and the negative control mAb T40/25 were absorbed to wells of a microtiter plate (1st Ab) and used to capture antigens from NP-40 lysates of HPB-ALL cells (hatched bars) or A α JY cells (solid bars). Radioiodinated second mAb were then added to detect antigens bound by the capture antibodies.

liver DPP IV after SDS-PAGE [31]. To determine if mAb AC7 would react with DPP IV, lysates of IL-2/OKT3 activated T cells were subjected to five sequential immunoprecipitations with the mAb OKT4, T40/25, AC7, or OKT3. The immunodepleted lysates were resolved by SDS-PAGE and transferred to EOM (Fig. 3). Lysates immunodepleted with OKT4, OKT3, or

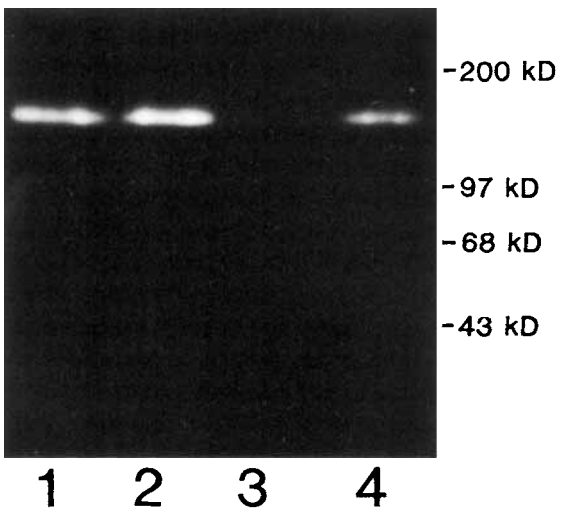

Fig. 3. mAb AC7 depletes DPP IV activity from the lysates of activated lymphocytes. Material remaining after exhaustive immunodepletion of Nonidet P-40 lysates of IL-2 + anti-CD3 activated lymphocytes was resolved by SDS-PAGE and transferred to EOM. Depletions were with OKT4 (**lane 1**), T40/25 (**lane 2**), AC7 (**lane 3**), and OKT3 (**lane 4**).

T40/25 retained DPP IV activity (Fig. 3, lanes 1,2,4) while immunodepletion with mAb AC7 totally removed activity (Fig. 3, lane 3). The immune complexes obtained from the first depletion step were also analyzed for DPP IV activity. Only the immune complexes obtained with mAb AC7 contained the enzymatically active molecule (data not shown).

Walborg et al. [25] have shown that heating to 100°C for 5 minutes results in an alteration in the structure of rat liver DPP IV from an enzymatically active M_r 150,000 form to an inactive M_r 105,000 form. Figure 4 shows SDS-PAGE autoradiographic (section A) and enzymatic analysis (section B) of the mAb AC7-reactive protein expressed on activated PBMC. When heated to 100°C the mAb AC7-reactive molecule migrated at a relative molecular weight of 105,000 in the presence (Fig. 4A, right panel, lane 1) or absence (Fig. 4A, left panel, lane 1) of reducing agent. Immune complexes treated for the same length of time at room temperature resolved into two bands of M_r 150,000 and 105,000 (Fig. 4A, left and right panels, lane 2). The DPP IV^-, $Ta1^-$ cell line HPB-ALL did not contain any mAb AC7-reactive material (Fig. 4A, left and right panels, lanes 3, 4). Prior to preparation for autoradiography, the gels were incubated with EOM paper to assay DPP IV activity (Fig. 4, Section B). Whole cell lysates of activated PBMC contained DPP IV activity that migrated at a M_r 150,000

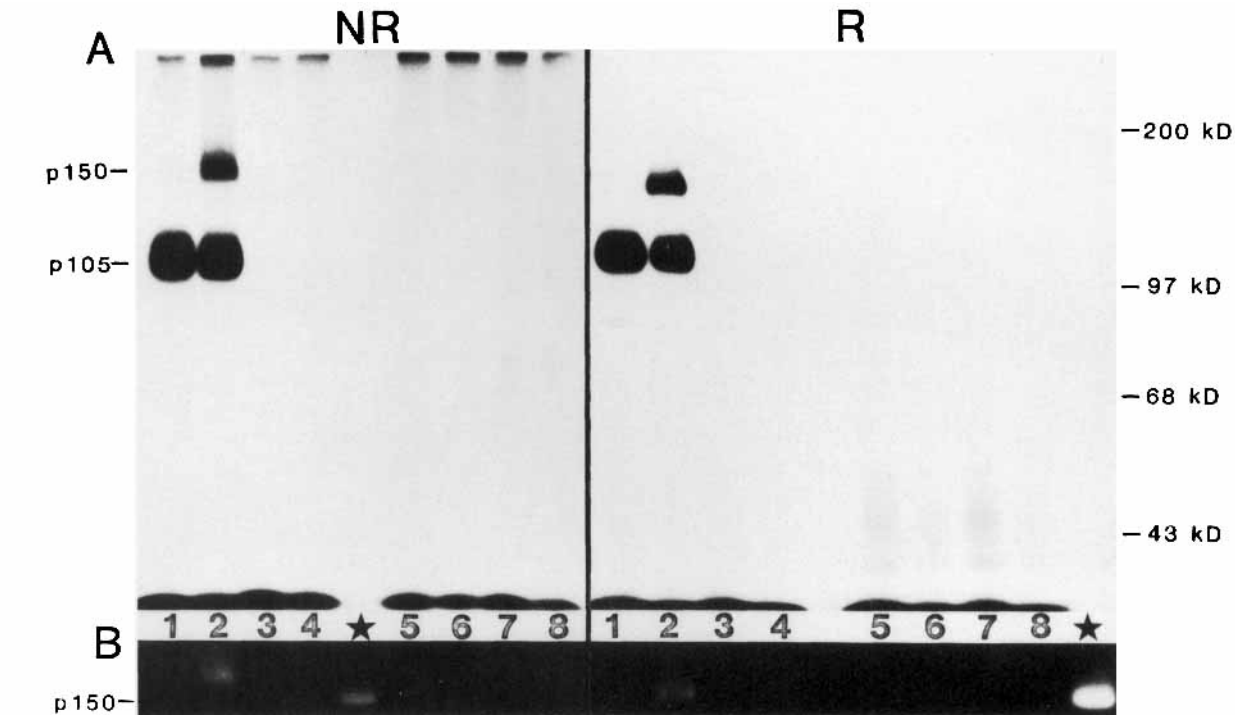

Fig. 4. Immunoprecipitation of DPP IV by mAb AC7. Cell surface radioiodinated HPB-ALL (**lanes 3,4,7,8**) and lymphocytes activated with anti-CD3 mAb + IL-2 (**lanes 1,2,5,6**) were lysed in NP-40 and immunoprecipitated with mAb AC7 (**lanes 1–4**) or the irrelevant isotype matched mAb D2 (**lanes 5–8**). The immunoprecipitates were resuspended in Laemmli sample buffer in the presence (right panel, R) or absence (left panel, NR) of the reducing agent 2-ME. The samples were then incubated for 5 minutes at room temperature (lanes 2,4,6,8) or 100°C (lanes 1,3,5,7) and resolved by 7.5% acrylamide SDS-PAGE. Lanes indicated with stars represent whole cell lysates of unlabeled anti-CD3 mAb + IL-2 activated T cells. Before preparation for autoradiography the gels were placed against EOM to detect DPP IV activity. Section **A** shows the autoradiographs and section **B** the EOM analysis of the same gel.

(Fig. 4B, ★ lanes). This activity was destroyed by heating to 100°C (data not shown). Dipeptidase activity was also seen in mAb AC7 immunoprecipitates from activated PBMC (Fig. 4B, lane 2, left and right panels) but not from HPB-ALL cells (Fig. 4B, lanes 3, 4). Since we have shown that enzyme activity correlated only with the M_r 150,000 form of the molecule (Fig. 3) only that portion of the EOM paper has been shown in Fig. 4. DPP IV activity was found at a slightly higher apparent relative molecular weight in experiments where immunoprecipitates were not reduced (Fig. 4B, left panel, lane 2). We believe this is due to retardation of DPP IV mobility by nonreduced M_r 150,000 antibody molecules. These experiments indicate that mAb AC7 reacts with both CD26 and DPP IV, and that DPP IV activity resides in a heat sensitive M_r 150,000 form of the CD26 molecule and not the M_r 105,000 form historically used to describe the CD26 molecule.

Ta1 has been shown to be expressed on activated T cells and the T cell tumor CCRF-HSB-2 but not on other T and B cell lines or on myeloid and monocytic lines. To determine whether the pattern of Ta1 expression correlated with DPP IV activity we screened a series of cell lines, and PBMC that had been activated by a variety of protocols. NP-40 lysates were resolved by SDS-PAGE and transferred to EOM. A single band of DPP IV activity at M_r 150,000 was detected in the lysates of PBMC activated by either PHA, alloantigen, IL-2, or IL-2 plus anti-CD3, as well as lysate from the T cell line CCRF-HSB-2 (Table I).

Monoclonal Anti–DPP IV Antibodies AC7 and Anti-Ta1 Potentiate PMA-Induced PBMC Proliferation

Work from other laboratories suggests that $CD26^+$ cells may have an important role in lymphocyte activation and proliferation [18,26, 27,30,44–47]. To assess the effects of monoclonal anti–DPP IV antibodies on lymphocyte proliferation, peripheral blood mononuclear cells (PBMC) were cultured in the presence of soluble

TABLE I. Cell Associated DPP IV Activity*

Cell	DPP IV
B cell lines	
PALLY	−
TOTO	−
SWEIG	−
SGAR	−
T cell lines	
HPB-ALL	−
MOLT-4	−
Jurkat	−
CCRF-HSB-2	+
Erythroleukemia	
K562	−
HEL	−
PBMC (activated with)	
Unactivated	−/+[a]
PHA	+
alloantigen	+
IL-2	+
anti-CD3 + IL-2	+

*Cells were lysed in NP-40 lysis buffer at 5×10^7 cells/ml and equal aliquots of each lysate were assayed for DPP IV activity as described in Materials and Methods.
[a]In some preparations of unactivated PBMC very faint DPP IV activity was seen.

mAb AC7 or mAb anti-Ta1 (Table II). Whereas these antibodies by themselves had no effect, lymphocyte proliferation induced with the phorbol ester PMA, an activator of protein kinase C, was dramatically enhanced by either of the two anti-CD26 mAb (Table II). The synergy between PMA and anti-CD26 mAb was very similar to that seen with a combination of anti-CD3 and PMA.

Table III demonstrates that the anti-CD26 enhancement of PMA-induced proliferation required the presence of a population of non-T cells. In the absence of this other population,

TABLE II. Human PBMC Proliferation Induced by Anti-CD26 mAb (^{3}H-thymidine Incorporation [cpm $\times 10^{-3}$])*

mAb (Specificity)	Nil	PMA
Control	0.3 ± 0.4	20.1 ± 1.9
OKT3 (CD3)	2.0 ± 0.7	98.8 ± 4.2
AC7 (CD26)	0.7 ± 0.5	92.3 ± 4.6
anti-Ta1 (CD26)	0.4 ± 0.2	90.7 ± 4.4

*Human PBMC (50,000 cells/well) were cultured in the absence or presence of PMA (10.0 ng/ml) plus mAb ascites; OKT3 (1/600), AC7 (1/300), anti-Ta1 (1/300). Cells were harvested after 4 days incubation with 1 uCi/well of ^{3}H-thymidine during the last 24 hours.

TABLE III. Soluble Anti-CD26 mAb Do Not Induce or Potentiate the Proliferation of Purified T Cells (^{3}H-thymidine Incorporation [cpm $\times 10^{-3}$])*

mAb	Nil	PMA
Control	1.4 ± 1.3	0.4 ± 0.1
OKT3	0.5 ± 0.4	62.7 ± 11.4
AC7	0.4 ± 0.0	4.8 ± 0.4
anti-Ta1	0.3 ± 0.0	1.5 ± 0.1
L25	1.9 ± 0.0	1.7 ± 1.2

*T cells were isolated from PBMC as described in Materials and Methods. The cells (50,000/well) were cultured in the presence or absence of 10 ng/ml PMA plus 1/300 dilution of mAb ascites. The cells were harvested after a four day incubation.

neither PMA nor anti-CD3 mAb induced purified T cells to proliferate, but a combination of PMA and anti-CD3 mAb resulted in a vigorous proliferation of purified T cells. However, only minimal proliferation occurred when PMA was mixed with the anti-CD26 mAb AC7, with mAb anti-Ta1, or with the isotype matched anti-VLA-4 mAb L25.

mAb AC7 was titered against a constant amount of phorbol ester in PBMC proliferation assays (Fig. 5A). In the absence of PMA, mAb AC7 was incapable of inducing proliferation. However, the addition of increasing amounts of mAb AC7 to a culture containing 10 ng/ml PMA resulted in increasing levels of lymphocyte proliferation. The optimal mAb AC7 concentration was between 500 and 1,000 ng/ml (Fig. 5A and data not shown). PMA was also titrated against 1 ug/ml of mAb AC7 (Fig. 5B). At 1 ng/ml PMA did not induce proliferation even in the presence of mAb AC7. At concentrations of 2.5 ng/ml, or more, PMA induced measurable proliferation which was significantly enhanced by mAb AC7. As expected, each increase in concentrations of PMA above 1 ng/ml increased lymphocyte proliferation. However, in the presence of 1 ug/ml of the AC7 antibody, proliferation was optimized and no significant differences in the levels of proliferation were seen with increasing concentrations of PMA over 2.5 ng/ml.

Binding of Immobilized mAb AC7 (Anti-CD26) to Freshly Isolated T Cells Enhances Proliferation

The effects of both soluble and immobilized mAb AC7 on the proliferation of purified T cells were tested and the results of a representative experiment shown in Table IV. The mAb T40/25, either soluble or immobilized, gave results

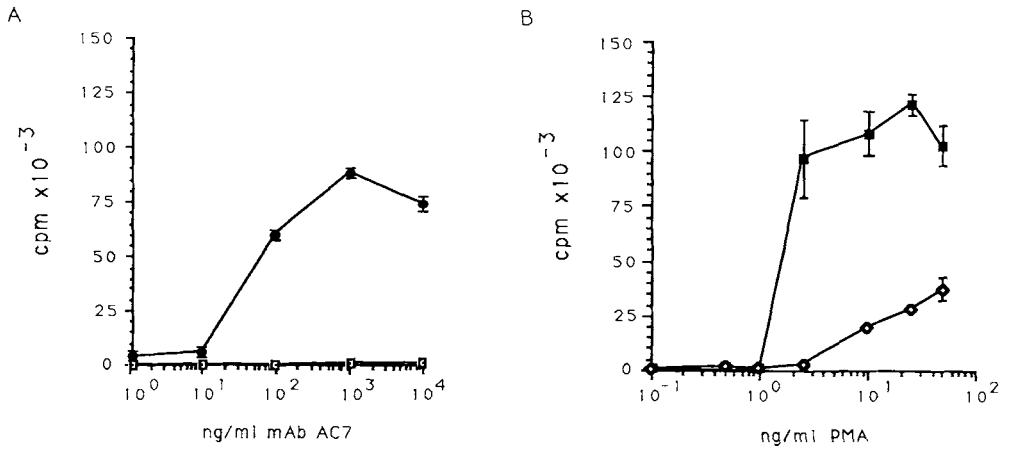

Fig. 5. Titration of the effects of mAb AC7 and PMA on PBMC proliferation. Human PBMC were isolated from buffy coats as described in Materials and Methods and cultured at a concentration of 50,000 cells/well in a 96-well tissue culture plate. PMA and/or mAb AC7 were added at the indicated concentrations to a final volume of 300 ul/well. The cells were harvested after 4 days with 1.0 uCi/well ^{3}H-thymidine during the final 24 hours of culture. **A:** ⊡ titration of mAb AC7 alone. ● titration of mAb AC7 in the presence of 10 ng/ml PMA. **B:** ◇ titration of PMA alone. ■ titration of PMA in the presence of 1 ug/ml mAb AC7.

indistinguishable from those obtained from wells coated with only bovine serum albumin or wells containing no soluble antibody (data not shown). Since mAb T40/25 had no effect on proliferation, columns and rows labeled T40/25 represent the control treatment.

In the absence of phorbol ester, sub-mitogenic concentrations of immobilized OKT3 (defined in the table by the column labeled Soluble mAb:

TABLE IV. T Cell Proliferation Induced by Soluble and Immobilized Anti-CD3 and Anti-CD26 mAb*

Immobilized mAb	Soluble mAb		
	T40/25	AC7	OKT3
Panel A			
T40/25	1.4 ± 1.3	0.8 ± 0.5	0.7 ± 0.3
AC7	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
OKT3	0.9 ± 0.3	3.9 ± 0.8	0.7 ± 0.3
OKT3 + AC7	38.6 ± 5.1	87.2 ± 8.4	63.6 ± 0.1
Panel B			
T40/25	0.4 ± 0.1	4.3 ± 0.3	53.4 ± 3.3
AC7	0.5 ± 0.0	5.7 ± 1.2	36.7 ± 2.1
OKT3	26.4 ± 5.1	50.5 ± 4.5	53.3 ± 1.8
OKT3 + AC7	27.2 ± 2.6	35.8 ± 2.6	28.8 ± 3.1

*Human T lymphocytes were purified and mAb immobilized to 96-well microtiter CovaLink plates as described in Materials and Methods. Soluble mAb was used as ascites at a final dilution of 1/1,200. T cells (50,000/well) were harvested after 6 days incubation. Results are expressed as mean ± standard deviation (cpm × 10^{-3}) of ^{3}H-thymidine incorporation from triplicate wells. **Panel A:** Antibodies alone. **Panel B:** Antibodies in the presence of 10 ng/ml PMA.

T40/25 and the row labeled Immobilized mAb: OKT3 [Table IV, panel A]), and saturating concentrations of soluble OKT3 (Table IV, panel A, Soluble mAb: OKT3 and Immobilized mAb: T40/25), were unable to induce purified T cells to proliferate. The addition of PMA, which by itself was not mitogenic for purified T cells, to soluble or immobilized mAb OKT3 resulted in proliferation (Table IV, panel B). Neither soluble nor immobilized mAb AC7 induced purified T cells to proliferate in the absence of PMA (Table IV, panel A). In the presence of PMA, saturating concentrations of soluble mAb AC7 induced a low level of proliferation, but immobilized mAb AC7 did not (Table IV, panel B). In the absence of PMA, neither mAb OKT3 nor mAb AC7 individually could induce proliferation, but when co-immobilized in the same well mAb OKT3 and mAb AC7 induced T cell proliferation (Table IV, panel A, Soluble mAb: T40/25). The proliferation caused by co-immobilized mAb AC7 and mAb OKT3 was increased by either soluble mAb AC7 (125% increase) or soluble mAb OKT3 (65%) (Table IV, panel A). The proliferation induced by the combination of PMA and immobilized mAb OKT3 was not enhanced by immobilized mAb AC7 but was enhanced by soluble mAb AC7 (Table IV, panel B). Interestingly, the proliferation resulting from treatment with PMA and saturating concentrations of soluble mAb OKT3 was inhibited by immobilized mAb AC7 (Table IV, panel B).

TABLE V. Effects of Soluble and Immobilized Anti-CD3 and Anti-CD26 mAb on Previously Activated Peripheral Blood Lymphocytes*

Immobilized mAb	Soluble mAb		
	T40/25	AC7	OKT3
Panel A			
T40/25	1.7 ± 0.0	1.6 ± 0.2	4.5 ± 0.6
AC7	2.5 ± 0.4	2.5 ± 0.2	22.3 ± 3.1
OKT3	24.6 ± 4.0	25.3 ± 1.7	24.9 ± 2.8
OKT3 + AC7	44.3 ± 2.1	41.2 ± 0.6	42.3 ± 3.6
Panel B			
T40/25	2.5 ± 0.9	3.2 ± 1.6	8.1 ± 0.1
AC7	4.6 ± 0.8	4.2 ± 0.2	10.6 ± 1.0
OKT3	7.4 ± 0.8	6.2 ± 0.4	9.7 ± 2.4
OKT3 + AC7	12.8 ± 1.4	11.2 ± 0.8	14.2 ± 1.8

*PBMC were activated with OKT3 and cultured for 22 days in the presence of 250 U/ml IL-2 as described in Materials and Methods. mAb were immobilized to 96-well microtiter CovaLink plates as described in Materials and Methods. The T cells (50,000/well) were harvested after incubation for 6 days in the presence of 1/1,200 dilution of soluble mAb ascites. Results are expressed as mean ± standard deviation (cpm × 10^{-3}) of ^{3}H-thymidine incorporation from triplicate wells. **Panel A:** Antibodies alone. **Panel B:** Antibodies in the presence of 10 ng/ml PMA.

Interaction of Immobilized mAb AC7 (Anti-CD26) With Previously Activated T Cells Allows These Cells to Respond to Soluble mAb OKT3, a Normally Non-Proliferative Signal

We also studied the effects of mAb AC7 and mAb OKT3 on the proliferation of previously activated lymphocytes. Immobilized mAb OKT3, at a concentration that did not induce proliferation in PBMC-derived T cells alone significantly stimulated the proliferation of T cells that had been growing for 22 days in the presence of IL-2. The proliferation induced by co-immobilized mAb OKT3 and mAb AC7 was approximately twice that induced by immobilized mAb OKT3 alone (Table V, panel A). Neither soluble nor immobilized mAb AC7 was capable by itself of inducing proliferation (Table V, panel A). Soluble mAb OKT3 had only a minor effect on the proliferation of previously activated T cells and did not significantly inhibit, or enhance, the proliferation caused by immobilized OKT3, or OKT3 immobilized with mAb AC7 (Table V, panel A). Interestingly, although immobilized mAb AC7 or soluble mAb OKT3 by themselves stimulated little, if any, increase in the proliferation of activated T cells, a combination of soluble mAb OKT3 and immobilized mAb AC7 resulted in a level of proliferation that was approximately equal to that generated by immobilized mAb OKT3. The co-stimulatory effect of soluble OKT3 and immobilized AC7 was not seen using unstimulated T cells (compare panel A in Tables IV and V). The addition of PMA to previously activated T cells generally resulted in decreased proliferation from that induced by the antibodies themselves but did not alter the overall pattern of mAb co-stimulation (Table V, panel B).

DISCUSSION

In this report we have described a new mAb, AC7, which by sequential immunoprecipitation and double determinant analysis was found to react with the same molecule as the CD26-specific mAb anti-Ta1 but at a spatially distinct site. A number of separately described, approximately M_r 105,000, T cell surface molecules may be equivalent to the CD26 antigen. The mAb CB.1, also known as anti-Tp103 [45], has been reported to react with a spatially distinct epitope on the same molecule bound by the mAb anti-Ta1 [46]. The equivalence of the protease DPP IV with CD26 is suggested by mAb binding studies [29], the observation that affinity chromatography using the anti-Tp103 mAb CB.1 co-purifies DPP IV activity [30], and cytochemical studies showing that, in addition to T cells, the CB.1 epitope is expressed on a wide range of non-lymphoid tissues [30], as would be expected if anti-CD26 mAb react with DPP IV. Recently a new mAb, 1F7, has been shown to deplete all material reactive with mAb anti-Ta1 from lysates of surface labeled $Ta1^{+}$ cells [47]. In contrast, depletion with anti-Ta1 did not remove all of the mAb 1F7-reactive material. Furthermore, the 1F7 antigen was expressed on such Ta1 negative peripheral blood cells as null cells, monocytes, and some B cells, as well as Ta1 negative B cell and hematopoietic cell lines [47]. The pattern of 1F7 expression is more complex than the patterns obtained with the CD26/DPP IV–reactive mAb Ta1 and CB.1. In cell surface binding studies mAb 1F7 almost totally blocked mAb anti-Ta1 binding, but mAb anti-Ta1 only partially blocked mAb 1F7 binding [47], suggesting that either the two epitopes are physically near one another, or that the binding of mAb 1F7 suppresses the expression of the Ta1 epitope. It remains to be elucidated whether the results of these structural and cytochemical analyses indicate alternate structural conformations of CD26 on different cell types, multiple members

of a gene family, or the association of multiple molecules into a complex cell surface structure.

By utilizing a novel enzyme overlay membrane system [31,41,42] we have determined that the cell surface molecule bound and immunoprecipitated by mAb AC7 exhibited the same enzymatic activity as the protease dipeptidyl peptidase IV (DPP IV), strengthening the observation of Hegen et al. [30]. We extended our analysis to include a correlation of DPP IV structure and function based upon what has been described in the rat liver [25,31]. As was expected from the rat studies, the material immunoprecipitated by mAb AC7 contained molecules of M_r 150,000 and 105,000 when samples were not boiled before analysis by SDS-PAGE (Fig. 4A). Analysis with EOM showed that DPP IV enzymatic activity resided exclusively in the M_r 150,000 band (Fig. 4B). When the immunoprecipitates were boiled, the M_r 150,000 band disappeared, along with DPP IV activity. These results indicate that human lymphocyte-derived DPP IV is structurally and functionally similar to that found in rat liver.

Walborg et al. [25] have shown that the enzymatically active site resides in the M_r 105,000 polypeptide but only the M_r 150,000 form of DPP IV exhibits activity. The reason for the dramatic change in the observed M_r of DPP IV after boiling is unknown. It is possible that DPP IV contains an intramolecular bond that is broken after heating resulting in an alteration in the migration of the molecule in SDS-PAGE but not in the absolute molecular weight of the molecule. Another possibility is that DPP IV is a heterodimer consisting of an approximately M_r 40,000 polypeptide covalently associated with the M_r 105,000 polypeptide through a heat-labile bond. If there is a M_r 40,000 polypeptide it seems to be either an intracellular molecule, or very difficult to label by cell surface iodination. Of interest in this regard is the observation of a M_r 50–70,000 molecule that, in some instances, was seen in mAb 1F7 immunoprecipitates of ^{125}I-labeled cell surface proteins from activated T cells [47].

Recently, investigations of human T cell associated DPP IV and the Ta1 antigen have indicated that CD26 may play a role in regulating lymphocyte activation and proliferation. Strong correlative evidence has been presented indicating that IL-2 secreting cells arise from the DPP IV$^+$ T cell population [27,28,44]. It has been suggested that the presence or absence of the Ta1 epitope is a useful marker in subdividing T cells into functional sub-populations. Hafler et al. [18] have shown that Ta1$^+$ cells, but not Ta1$^-$ cells, generate secondary immune responses to antigens such as tetanus toxoid and mumps virus and have presented evidence that Ta1$^+$ cells may have fewer, or lower, threshold signaling requirements than Ta1$^-$ cells [18,20].

The T cell proliferation initiated by IL-2, PMA, anti-CD2 mAb, or anti-CD3 mAb can be amplified by mAb reactive with a wide range of T cell surface molecules [48–56] suggesting that a large number of cell surface molecules may play a role in modulating lymphocyte activation and/or proliferation. Similar results are now being obtained for anti-CD26 mAb. In the presence of accessory cells mAb CB.1 (anti-Tp103) and anti-Ta1 have been shown to increase IL-2-induced T cell proliferation [45,46]. The proposed mechanism for this enhancement is through stimulation of the IL-2 autocrine growth pathway, resulting in increased IL-2R expression and IL-2 secretion [45,46]. Also, mAb 1F7 has been shown to co-stimulate either anti-CD2 or anti-CD3 mAb-induced T cell proliferation increasing IL-2 secretion and IL-2R expression [57]. We found that mAb AC7 also stimulated T cell proliferation but fell between mAb anti-Ta1 and mAb 1F7 in terms of required co-signals. When co-immobilized to plastic with anti-CD3 mAb both mAb AC7 and mAb 1F7, but not anti-Ta1 mAb, were capable of co-stimulating proliferation of purified T cells (ref. 57 and Tables IV and V). However, while adding phorbol ester to immobilized mAb 1F7 caused T cells to proliferate, neither mAb anti-Ta1 nor AC7 were capable of doing the same (ref. 57, Tables IV and V, and data not shown). Therefore, although cytochemical and immunochemical studies placed mAb AC7 closer to mAb anti-Ta1 than to mAb 1F7, some functional requirements were similar to those of mAb anti-Ta1 while others mimicked mAb 1F7.

A potential mechanism to explain anti-CD26 mAb stimulated T cell proliferation would be that mAb binding to CD26$^+$ cells induces a direct proliferative signal to the T cell. Support for this mechanism arises from numerous studies using mAbs reactive with many cell surface antigens. Binding of mAbs to some T cell surface antigens results in a rapid increase in intracellular free Ca^{++} [58–60], changes in intracellular pH [61], inositol phosphate turnover [62,63], translocation of protein kinase C from the cytosol to the

plasma membrane [64], regulation of cAMP levels [65], and protein phosphorylation [66], all of which are believed to be primary events in cellular activation. The observations made using anti-CD26 mAb leave open the possibility that CD26 acts directly as an intracellular signal transducer when interacting with its ligand. However, some other signaling event must occur in conjunction with anti-CD26 stimulation such as the presence of phorbol ester, anti-CD3 mAb, and/or accessory cells.

An alternative possibility to a direct intracellular signalling mechanism would be that anti-CD26 mAb indirectly influences lymphocyte proliferation through altering the enzyme kinetics of DPP IV. A number of proteins involved in lymphocyte biology are potential substrates for DPP IV, including IL-1, IL-2, and lymphotoxin [67,68]. Schön and colleagues have shown that DPP IV–specific substrate inhibitors and polyclonal anti–DPP IV antiserum will block mitogen stimulated T lymphocyte proliferation, IL-2 and interferon secretion, and the synthesis of immunoglobulin by B cells in response to the T cell dependent mitogen PWM [44,67,68]. The possibility that certain lymphokines are substrates for DPP IV is supported by the following observations. DPP IV from liver is capable of removing N terminal dipeptides from substance P, a small (11 amino acid) polypeptide neurotransmitter [69] and promilletin, the lytic protein from honey bee venom [70]. Also, DPP IV isolated from pancreas, or placenta, is capable of removing amino-terminal dipeptides from the enzymes trypsinogen and pro-colipase [71]. In the case of pro-colipase, a second dipeptide is removed after the initial hydrolysis, resulting in a partial activation of colipase enzymatic activity. However, as yet, no direct evidence has been presented that lymphokines are substrates for T cell DPP IV.

We have discovered that the binding of mAb AC7 to the cell surface of freshly isolated T cells could result in either stimulation or inhibition of proliferation depending either on the way the antibody was presented, or on the presence of accessory signals (Table IV). The complexity of mAb AC7 mediated stimulation, or inhibition, of T cell proliferation is now being studied.

An interesting observation made during the course of these studies was the different requirements for mAb AC7/OKT3 co-stimulation between freshly isolated and cultured T cells. To co-stimulate freshly isolated peripheral blood T cells mAb AC7 needed to be immobilized together with mAb OKT3 (Table IV). However, T cells maintained in culture with IL-2 were stimulated by immobilized mAb AC7 in conjunction with soluble mAb OKT3, signals that by themselves were non-mitogenic (Table V). It has been reported that DPP IV can bind to fibronectin and that this interaction is promoted by competitive peptide inhibitors of DPP IV [31]. One possibility is that, for activated and memory T cells, which express high levels of DPP IV, the binding of DPP IV to ligand may cause the enzyme to bind to the extracellular matrix, or in some cases extracellular matrix proteins themselves may be ligands for DPP IV. The binding of CD26 to matrix could generate a signal that would allow the T cell receptor complex to respond to antigen presented in a sub-mitogenic context. Another potential extracellular matrix ligand for DPP IV is collagen [32,33] and recently it was shown that in serum free conditions a mixture of co-immobilized collagen and suboptimal doses of anti-CD3 mAb induced proliferation among $CD4^+$ T cells [72]. The proliferation induced by co-immobilized collagen and anti-CD3 was almost totally inhibited by mAb 1F7, but not by mAb anti-Ta1 which is also an anti-CD26 mAb. One obvious interpretation for these results would be that mAb 1F7, but not mAb anti-Ta1, reacted with the collagen binding epitope on CD26. However, mAb specific for the β1 subunit (CD29) of integrins or the α3 subunit (CD49c) of the integrin VLA-3 could also almost totally inhibit proliferation. Since the ability of mAb 1F7 to block the binding of $CD4^+$ T cells to collagen was not studied these observations leave open a second possibility, that the binding of soluble mAb 1F7 to the CD26 protein resulted in a regulatory signal independent of collagen binding. This second possibility is supported by observations that CD26 binding to collagen, at least for rat liver DPP IV, is mediated through an indirect interaction of CD26 with fibronectin which has interacted with the collagen [31].

The sequelae of T cell activation are numerous and complex [10,73,74] and include a cascade of intracellular second messages [73] which influence changes in the levels of expression of numerous intracellular and extracellular proteins, including CD26/DPP IV [10,74]. Therefore, the ability of CD26 to influence T cell proliferation could arise by a number of different mechanisms, or combination of mecha-

nisms. One possibility is that primary activation events may induce a state of responsiveness in the T cells toward signals generated through CD26. It is also possible that the increased expression of CD26 on activated and memory T cells enhances an otherwise minimal signal. Another possibility is that after T cell activation the structure of CD26 might be modified to allow the generation of secondary signals. Precedent for activation-mediated modifications has been seen with the integrin LFA-1 (CD11a/CD18). Stimulation of lymphocytes with phorbol esters, anti-CD2, or anti-CD3 will induce adhesive function in this molecule, possibly through phosphorylation of the CD18 subunit [75]. A likely possibility is that a combination of changes in the responsive state of the cell and in the expression, structure, and function of CD26 will be involved in allowing this molecule to influence the activation and proliferation of the T lymphocyte.

Considering the complicated nature of anti-CD26 mAb effects on T cell proliferation further studies are necessary to determine if all of the antibodies described as CD26-specific actually recognize the same polypeptide, as well as which of the possible mechanisms, or combination of mechanisms, are responsible for the observed effects by anti-CD26 mAb on lymphocyte proliferation.

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