Targeting of an Activated T-Cell Subset Using a Bispecific Antibody-Toxin Conjugate Directed Against CD4 and CD26

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We have developed a bispecific antibody that recognizes the CD4 and CD26 antigens simultaneously and that was examined for its ability to target CD4+CD26+ T cells. These latter cells constitute the activated component of the CD4+CD29+CD26+ memory T-cell subset that provides help for B-cell Ig synthesis and help for responses against recall antigens. The purified bispecific antibody exhibited an estimated dissociation constant \(k_d\) of \(2.4 \times 10^{-9}\) mol/L on comparison with 1.1 \(\times 10^{-9}\) mol/L for anti-CD26, and 1.6 \(\times 10^{-10}\) mol/L for anti-CD4. Surface plasmon resonance was used to show the bifunctional capacity of the antibody. On binding \(^{125}\text{I}-\text{bispecific antibody to phytohemagglutinin (PHA)-activated T cells, 54.4}\%\) of the bound antibody was internalized. This was the result of bispecific binding, because monovalent fragments of anti-CD4 and anti-CD26 were not able to modulate antigen or induce internalization using both a fluorescent assay and an \(^{125}\text{I}-\text{internalization assay}. The ability of the bispecific antibody to be internalized was used to deliver a toxin, blocked ricin, specifically to cells that are CD4+CD26+. The inability of monovalent fragments to be internalized formed the basis for our hypothesis that monovalent binding by the bispecific immunotoxin would not result in internalization. Against resting E+ T cells, the bispecific immunotoxin developed a minimal effect. On preactivating the same cells, using phorbol myristate acetate (PMA)/ionomycin on concanavalin A (ConA) or especially PHA, levels of CD26 were upregulated and the immunotoxin effectively inhibited the ability to provide help for B-cell Ig synthesis while leaving intact the CD4+CD26+ and CD4+CD26− populations; an effect observed both functionally and by phenotype. The bispecific antibody proved to be most effective at inhibiting a heterologous mixed leukocyte reaction. We propose that this reagent may form the basis for the rational design of toxins designed to modulate activated T cells from, or directed against, tissue grafts.

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modified to block resident lectin sugar-binding capacity (blocked ricin, bRicin), and tested the immunoconjugate for its ability to target activated CD4*CD26+ T lymphocytes specifically and simultaneously spare CD4*CD26− T cells.

**MATERIALS AND METHODS**

**Isolation of lymphoid populations.** Human peripheral blood leukocytes (PBL) were isolated from the blood of healthy donors by centrifugation over Ficoll-Hypaque (Pharmacia, Piscataway, NJ). The PBL were separated into E rosette-positive (E+) and E rosette-negative (E−) populations after incubation with 5% sheep red blood cells (SRBC) (Microbiological Associates, Bethesda, MD) as previously described.20 E+ cells were further depleted of monocytes by adherence, and the T cells thus obtained were greater than 95% CD3+. For some experiments, the E− PBL were further separated to yield a CD4+-enriched population. In this case, E− PBL were incubated with 5 μg/mL/10⁶ cells of anti-CD20 (Clone B1), of anti-CD16 (clone 3G8) and of anti-CD8 (Clone 7t3F9) for 30 minutes at 4°C in RPMI 1640 + 10% pooled human male AB serum. After two washes in the same medium, the cells were incubated at a 1:5 ratio with anti-isotype Ig-coated magnetic beads (Advanced Magnetics, Cambridge, MA) for a further 30 minutes at 4°C, after which the cell-bead complexes were removed with a magnet to yield a cell population composed of greater than 95% CD4+ cells.

**Cell lines.** The parental hybridoma cell lines used in this study were an anti-CD26 clone designated 1F7 (IgG1−κ), and an anti-CD4 clone designated 19thy5D7 (IgG2a−κ). The CD4*CD26+ T-cell clones were isolated from one of us (J.S.D.C.), who had been hyperimmunized with tetanus toxoid and the cells subsequently maintained by a cycle of stimulation with tetanus toxoid (1 μg/mL) (Connaught Laboratories, Swiftwater, PA) and autologous antigen-presenting cells followed by expansion in 10% T-cell growth factor (TCGF) and 10 U/mL recombinant interleukin-2 (rIL-2) (kindly provided by Dr S. Gillis, Immunex, Seattle, WA).

**Production of bifunctional antibodies.** The anti-CD4 clone 19thy5D7 was rendered resistant to G418 by transfecting the hybridoma with the plasmid sν2neo-SP (kindly provided by Dr Michel Streuli, Dana Farber Cancer Institute) using the electroporation technique. The cells surviving in 500 μg/mL G418 active metabolite (GIBCO, Grand Island, NY) were allowed to grow for 14 days, after which the wells were examined for clonal growth and the supernatants tested to establish that the cell maintained secretion of an antibody with properties similar to that of the anti-CD4 parent.

Before fusion, the G418-resistant 19thy5D7 cells were resuspended in cold Dulbecco’s modified Eagle’s medium (DMEM) on ice and exposed to 10 mmol/L iodoacetate for 30 minutes to inactivate the cells metabolically, followed by three washes. The inactivated cells (10⁷) were then fused with 10¹¹ 17B1 hybridoma cells in 1 mL of DMEM containing 45% polyethylene glycol (M, 1600) and 5% dimethyl sulfoxide (DMSO) (Sigma Chemical Co, St Louis, MO). Cells were left on ice for 30 minutes, washed, and resuspended in DMEM supplemented with 10% newborn calf serum and 10% thymoma-conditioned medium (Sigma) and then plated into 96-well flat-bottomed microtiter plates. After 1 day, G418 was added (500 μg/mL active metabolite), and the drug-containing medium was replaced every 2 days. Supernatants of wells containing clones that appeared were dialyzed 1:2 in phosphate-buffered saline (PBS) + 0.05% Tween 20 (PBS-Tween) and 100 μL was transferred to enzyme-linked immunosorbent assay (ELISA) plates (Costar, Cambridge, MA) that had been coated with goat anti-mouse IgG1 or anti-mouse IgG2a (10 μg/mL) (Fisher Biotech, Pittsburgh, PA). After incubation for 2 hours at room temperature, wells were washed three times with PBS-Tween and then incubated with polyclonal goat antimouse IgG isotype alkaline phosphatase conjugates (1:2000 in PBS-Tween, Fisher Biotech). After 2 hours, the wells were washed with PBS-Tween, and color development was initiated by addition of 150 μL of diethanolamine phosphate (1 mg/mL in 0.05 mol/L 3-[cyclohexylamino]-1-propanesulfonic acid [CAPS] buffer containing 1 mmol/L MgCl₂, pH 9.6). Absorption was determined at 405 nm using a Titertek Multiskan MC (Flow Labs Inc, McLean, VA) scanner. By using anti-lgG1 to coat the plates and developing with anti-lgG2a alkaline phosphatase, we could detect relative levels of expression of hybrid heavy chain isoforms, whereas parental heavy chain isoforms were detected by using the same polyclonal anti-isotype for binding and detection. Supernatants that tested positive for hybrid heavy chain (γ1/γ2a) were then tested for their ability to block the binding of fluorescein isothiocyanate (FITC)-labeled anti-CD26 (Ta1) (Coulter Immunology, Hialeah, FL) and phycoerythrin-labeled anti-CD4 (Coulter) to a T-cell clone expressing both these antigens.

**Preparation of radio-iodinated antibodies.** Preparations of the anti-CD4, anti-CD26, their respective F(ab') fragments, and bispecific antibody were iodinated by incubating 50 μg of antibody in 100 mmol/L phosphate buffer, pH 6.8, with one Iodobead (Pierce, Rockford, IL) for 5 minutes, followed by the addition of 1 μCi Na¹²¹I (NEN, Boston, MA) for 5 minutes to yield iodinated protein with specific activities ranging from 2.5 × 10⁶ to 10⁷ cpm/μg.

**Determination of antibody internalization.** Freshly expanded CD4*CD26+ T-cell clone cells in logarithmic phase of growth were incubated with ¹²¹I-labeled antibodies in RPMI 1640 medium supplemented with 10% pooled human male AB serum for 16 hours at 37°C and at 4°C (to provide a measure of background internalization). Each sample (2 × 10⁶ cells) was split and 10⁶ cells were stripped of membrane-bound antibody by incubation for 2 minutes in RPMI 1640 + 10% fetal calf serum (FCS), pH 2.9, whereas the
remaining 10⁶ cells were used as unstripped controls. The samples were counted in a gamma counter (LKB-Wallac, Turku, Finland) and the cycles per minute of untreated cells were assumed to represent total cell-associated antibody, whereas the cycles per minute of the stripped cells was assumed to represent only that antibody that had been internalized.²²

**Immunotoxin preparation.** Immunotoxins containing blocked ricin (Bricin) were prepared at ImmunoGen Inc (Cambridge, MA) as described elsewhere.23,24 Essentially, the galactose-binding sites on the B chain of intact ricin dimer were blocked with triantennary N-linked oligosaccharides derived from fetuin, which themselves contained a disulfide group that could be specifically reduced for conjugation to antibody without breaking the ricin A–ricin B chain disulfide link. Antibody modified with succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) (Pierce, Rockford, IL) to generate 0.5 maleimide groups per IgG molecule is then reacted with reduced blocked ricin and the conjugate is purified away from unreacted reagents by ion exchange chromatography on S-Sepharose followed by affinity chromatography using a monoclonal anti-bRicin column. Anti-CD19-bRicin (B4-bRicin) was kindly provided by Dr. R. Rasmussen, Dana Farber Cancer Institute.

**Binding dynamics using surface plasmon resonance.** Recombinant CD4 (Biogen) was immobilized on a BIAcore sensor (Pharmacia Biosensor AB, Uppsala, Sweden) coated with a dextran matrix as described elsewhere.25 Using a fine flow dynamics system within the BIAcore system, reagents can be passed over the immobilized CD4 and binding detected by measuring changes in the resonance angle that is dependent on surface refractive index, which is related to the mass of immobilized material. Recombinant soluble CD26 was kindly provided by Dr. T. Tanaka, Dana Farber Cancer Institute.

**Preparation of monovalent F(ab')₂ fragments of anti-CD4 and anti-CD26.** Six milligrams of anti-CD4 (clone 19thy5D7, IgG2a, Protein A-purified) and 6 mg of anti-CD26 (clone IF7, IgG1, protein A-purified) were equilibrated in 20 mmol/L phosphate, 10 mmol/L EDTA, pH 7.0 (phosphate-EDTA) and concentrated to 2.5 mg/mL. The antibody preparations were then supplemented with cysteine HCl (20 mmol/L final) and incubated with 0.5 mL of agaro-immobilized papain (Pierce) for 5 hours at 37°C with constant rotation. The digests were separated from the gel and were diluted 1:1 with Monoclonal Antibody Purification System (MAPS) II protein A-binding buffer (Bio-Rad). After passage through a protein A–Sepharose column, the nonbinding F(ab')₂ fragments were concentrated on a 10-Kd molecular weight cutoff centrifugal microconcentrator (Filtron, Northborough, MA) and equilibrated in PBS. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions revealed a single band of approximately 55 Kd for the F(ab')₂ fragments.

**Analysis and separation of lymphocyte subpopulations by flow cytometry.** Cytofluorographic separation of cell populations was performed using an Epics C flow cytometer (Coulter) as previously described.26 Routine fluorescence analysis for screening hybridoma and quadroma supernatants was performed using a Coulter Profile Flow Cytometer (Coulter, Hialeah, FL). Conditions of incubation consisted of 30 minutes on ice with a primary antibody in PBS + 5% human pooled AB serum (to reduce nonspecific binding through the Fc receptor), followed by three washes in the same medium, with a repetition of this procedure for indirect immunofluorescence. Cells that could not be analyzed immediately were fixed in PBS + 1% paraformaldehyde and stored at 4°C in the dark. For surface marker analysis, all antibodies were used at a concentration within the range 1 to 5 μg/mL.

**Proliferative responses of T lymphocytes.** E¹ BPL or subset-enriched T cells were resuspended to 10⁶/mL and were incubated with differing concentrations of immunotoxins at 37°C throughout the entire culture period. In some instances, before exposure to immunotoxins, the E¹ cells were preactivated for 3 days using ConA (20 μg/mL) (Sigma), phytohemagglutinin (PHA) (5 μg/mL), or phorbol myristate acetate (PMA) and ionomycin (2 and 300 ng/mL, respectively). Autologous adherent cells at 2.5% of total cell number were added to all cultures. After extensive washing, 10⁶ cells were added together with the mitogen PHA (5 μg/mL) and nonadherent cells (2,500 for 2.5% final) to each well in a 96-well round-bottomed plate. On the third day after initiation of the cultures (maintained at 37°C in a humidified 8% CO₂ environment), each well was pulsed with 1 μCi of [³H]thymidine (ICN Biochemicals, Irvine, CA). After overnight incubation, the cells were harvested and counted using the Pharmacia-LKB Betaplate 1205 system. For the heterologous mixed leukocyte reaction (HLMR), 50,000 responder PBL were mixed with 50,000 stimulator PBL (irradiated with 5,000 rad) in 200 μL complete RPMI in round-bottomed microtiter wells, and proliferation was assessed at 6 days by addition of [³H]thymidine as described above.

**Detection of intracellular secretion of IgG.** Nonadherent E¹ cells (10⁶) were cultured with 2,500 adherent E¹ cells, a 1:100 dilution of pokeweed mitogen (PWM) (GIBCO), and 5 × 10⁴ enriched CD4⁺ cells that had been pretreated with immunotoxins or control preparations. The cultures were performed in flat-bottomed 96-well microtiter plates and were incubated in a humidified 8% CO₂ atmosphere for 9 days, after which the supernatants were harvested, diluted 1:2 to 1:10 in PBS-Tween (0.01%), and incubated in flat-bottomed MicroELISA plates precoated with 10 μg/mL polyclonal antihuman Ig (Fisher BioTech). After 1 hour incubation at room temperature, the plates were washed three times in PBS-Tween and incubated with a polyclonal antihuman IgG-labeled phosphatase conjugate, and bound enzyme activity was determined as described above for screening hybridoma/quadroma supernatants.

**Determination of specific subset survival after exposure to immunotoxins.** PBL were incubated in RPMI + 10% pooled human male AB serum in the presence of anti-CD4–blocked ricin, anti-CD26–blocked ricin, or bispecific anti-CD4–anti-CD26–blocked ricin for 4 days at 37°C. At this time, the surviving viable cells were isolated by centrifugation over Ficoll-Hypaque and counted. The same samples were then incubated with a FITC-labeled anti-CD26 and phycocyanin-labeled anti-CD4 (Coulter) and the percentage of CD4⁺CD26⁺, CD4⁺CD26⁻, and CD4⁻CD26⁺ cells were determined as described above. The absolute numbers of phenotyped cells were then calculated from the percentage fluorescent of the total viable cell count for each sample.

**RESULTS**

**Separation, purification, and characterization of anti-CD4–anti-CD26 bispecific antibody.** We generated a quadroma secreting anti-CD4–anti-CD26 bispecific antibody by fusing parental hybridomas differing in their heavy chain isotypes, thus providing a handle for the subsequent purification of the hybrid heavy chain bifunctional antibody. A preparation that consisted of hybrid γ₁/γ₂a heavy chains and expressing functional CD4– and CD26–binding activity was isolated by initial elution with a pH gradient from protein A (Fig 1A), followed by separation on hydroxyapatite and elution with a phosphate gradient (Fig 1B). The material at this stage consisted of at least four separate entities, all of which were heavy-chain hybrids, so functional CD4–binding antibody was isolated by affinity binding and elution.
TARGETING OF ACTIVATED T CELLS

Fig 1. Chromatographic separation and purification of bispecific anti-CD4-anti-CD26 antibody. (A) Elution using a pH gradient of antibody bound to Sepharose—protein A (ProA). The hatched bar indicates the pooled fractions used for further separation. (B) The material isolated after ProA pH elution was dialyzed against 50 mmol/L phosphate, pH 6.8, and eluted from a hydroxylapatite HPLC column (dotted line; 50 to 350 mmol/L phosphate containing 1 mmol/L CaCl₂, pH 6.8). The heavy chain isoforms were identified by sandwich ELISA. The hatched bar indicates the material subsequently purified on a Sepharose-rCW column.

from rCD4 immobilized on Sepharose, with 31% of the initially applied material binding to the column. This material formed the basis for all studies and was used to prepare immunotoxins.

An approximation of binding affinity²¹ was obtained from the amount of antibody preparation required to give 50% of the maximal the binding to a CD4⁺CD26⁺ T-cell clone (Fig 2). This yielded the following dissociation constants: 1.6 × 10⁻¹⁰ mol/L for anti-CD4, 1.1 × 10⁻⁹ mol/L for anti-CD26, and 2.4 × 10⁻⁹ mol/L for the bispecific anti-CD4-anti-CD26. Using the bRicin conjugate of the bispecific antibody, the apparent kd increased nearly twofold to 4.1 × 10⁻⁹ mol/L. This effect of conjugation was also reported for another bRicin conjugate, anti-CD19–bRicin.²⁴ Identical results were obtained deriving the kd from the mean cell fluorescence values as opposed to percentage positive. The reduced affinity of the bispecific antibody may reflect in part some contamination by monovalent CD4-binding hybrid, not all of which would be separated from the bispecific antibody using the procedure we have devised. Despite the lower affinity of the bispecific antibody on comparison with its parental antibodies, at saturating concentrations, the amount of bispecific antibody binding to activated T cells was considerably higher than either of the parental antibodies alone (see Fig 5). The lower affinity also manifest itself in the weak ability of the bispecific antibody to compete with the parental antibodies in a fluorescence inhibition assay (Fig 3). Nevertheless, the specificity of the bispecific antibody was clearly manifest in its ability effectively to block the binding of both parental-type antibodies.

Demonstration of bispecific binding by surface plasmon resonance. After immobilizing CD4 (25 μg/mL, ~1 ng/mm² final surface concentration) on the biosensor surface, recombinant soluble CD26 (25 μg/mL) was passed over the surface (Fig 4, label A). Clearly, the CD26 does not bind to CD4 to any measurable degree in its own right. After washing through the flow system with 10 mmol/L HEPES, bispecific anti-CD4–anti-CD26 was passed through the flow cell and approximately 0.73 ng/mm² bound to the surface (Fig 4, label B). On a moles per liter basis, this gives a ratio of 0.25 mol of bispecific (160 Kd) bound per mole CD4 (55 Kd), which implies that not all the available immobilized CD4 was available for binding. After a moderate dissociation had occurred (the system is based on a dynamic equilibrium process), soluble CD26 (25 μg/mL) was passed over the surface resulting in 0.1 ng/mm² binding (Fig 4, label C). After accounting for molecular weight (110 Kd) for the CD26, 0.2 mol of soluble CD26 bound per mole bispecific antibody. Therefore, we may infer that at least 20% of our preparation is true bispecific antibody and, given the limitations of the system (binding of bispecific to the CD4-coated surface may sterically hinder simultaneous binding of CD26, as well as a possible 30% contamination by monovalent anti-CD4), it is likely that considerably more than 20% is actively bispecific. Monoclonal anti-CD4 bound to the immobilized rCD4 could not bind soluble CD26.

Modulation of CD4 and CD26 by anti-CD4-anti-CD26 bispecific antibody and internalization. For the purpose of delivering a toxin to the cell interior, actual binding affinity may be of less importance than the ability to be internalized after bivalent binding. Accordingly, we looked at the ability of the parental antibodies, their monovalent F(ab) fragments, and the bispecific antibody to bind to resting and PHA-activated T cells (Fig 5). First, neither the monovalent
we performed an assay using radioiodinated antibodies where, on binding to CD4\textsuperscript{+}CD26\textsuperscript{+} T cell clones cells, surface-bound antibody may be stripped from the membrane using low pH buffers, whereas internalized antibody will remain unaffected by such a procedure. Using \(^{125}\text{I-anti-CD4}\), 45.7\% \pm 4.5\% of cell-bound antibody was internalized. For \(^{125}\text{I-anti-CD26}\), 114.8\% \pm 7.9\% of cell-bound antibody was internalized, whereas 54.4\% \pm 3.3\% of cell-bound \(^{125}\text{I-labeled bispecific antibody was internalized}. In a separate experiment, using \(^{125}\text{I-anti-CD4 F(ab') fragment}, 14.1\% \pm 6.4\% of cell-bound monovalent anti-CD4 was internalized. For \(^{125}\text{I-anti-CD26 F(ab') fragment}, 9.3\% \pm 3.8\% of cell-bound monovalent anti-CD26 was internalized, whereas 42.3\% \pm 8.1\% of cell-bound \(^{125}\text{I-labeled bispecific antibody was internalized. This suggests that surface CD4 is the limiting factor for internalization by the bispecific antibody. Clearly, monovalent binding through the F(ab') fragments results in minimal internalization from which we may infer that the internalization of the bispecific antibody is dependent on its ability to bind to both its target antigens.}

Selective abrogation of CD4\textsuperscript{+} T-cell function by bispecific immunoconjugate. Having shown that the bispecific antibody can modulate and be internalized, we next wished to test whether this characteristic could be used specifically to deliver a toxin, blocked ricin, to a cell expressing both CD4 and CD26 and inhibit in vitro functions expressed by that cell. First, we measured the inhibition of the simple ability to proliferate to a mitogen (PHA), which is a property not restricted to CD4\textsuperscript{+}CD26\textsuperscript{+} cells (Fig 6). In this assay, where unstimulated E\textsuperscript{+} cells were exposed to the immunoconjugates for the entire 72-hour culture period, the order of potency of \textit{bRicin immunoconjugate was: anti-CD26 > anti-CD4 > bispecific anti-CD4-anti-CD26 (Table 1). On preactivating the target cells with PHA before exposure to the immunoconjugates, the efficacy of the bispecific anti-CD4-anti-CD26 increased slightly, and they increased slightly more if the cells were preactivated with PMA/ionomycin or ConA. Per-

F(ab') anti-CD4 nor the F(ab') anti-CD26 was modulated from the cell surface to any significant extent. On resting E\textsuperscript{+} cells, the surface expression of both the parental anti-CD4 and the anti-CD26 was considerably reduced after 18 hours of incubation at 37°C implying modulation, but under similar circumstances, the modulation by the bispecific antibody was minimal. However, on PHA-activated cells, a different picture emerged. The expression of CD4 was reduced, the intensity of expression of CD26 was increased, and at 37°C the bispecific antibody now effected a strong modulation represented by a fall in the mean cell fluorescence.

Modulation may be interpreted as internalization or shedding; thus, to differentiate between these mechanisms,
hapes representing the increase in CD26 expression on activation, in all instances the bispecific immunotoxin was more effective at inhibiting the PHA-induced proliferative response of preactivated T cells than it was at inhibiting resting T cells. Nevertheless, the response to these mitogens is a relatively unrestricted polyclonal event, and as such, we hypothesized that the bispecific immunotoxin would be less effective than the anti-CD4 and anti-CD26 immunotoxins. With one exception, this was true. In the instance of ConA preactivation, the bispecific immunotoxin was as efficacious as the anti-CD4 immunotoxin, perhaps implying a preferential expansion of non-CD4 T cells. Preactivation with PMA and ionomycin resulted in a higher nonspecific killing (as represented by the bRicin control) making it difficult to interpret whether increased inhibition of PHA proliferation was specific or was the result of a change in cell membrane properties. In one experiment, an immunotoxin directed against B cells rather than T cells (an anti-CD29-bRicin conjugate) was compared with the bispecific conjugate in ability to block PHA responses. The anti-CD19-bRicin conjugate was ineffective over the dose range 10^{-12} to 10^{-9} mol/L, which suggests that interactions through nonspecific Fc receptor binding were not of consequence (data not shown).

The cells that provide help for PWM-stimulated B-cell IgG synthesis predominantly express both CD4 and CD26, thus, exposure to the bispecific immunotoxin should result in as strong an inhibition of T-cell help for B-cell IgG synthesis as that effected by anti-CD4–bRicin or anti-CD26–bRicin. To test this notion, we incubated resting or activated CD4⁺ T cells with the immunotoxins and then assessed their ability to provide help for IgG synthesis (Fig 7). Using resting CD4⁺ cells, the anti-CD4 immunotoxin was most effective, with the bispecific toxin conjugate less effective by a factor of 10, and the anti-CD26 immunotoxin lying in between in terms of effectivity. After activation of the CD4⁺ cells before incubation with the immunotoxin, the generated blast cells were more sensitive to the bispecific immunotoxin (Table 1) irrespective of the mode of activation used, and in each instance the efficacy was as good or better than that observed with the anti-CD4 and anti-CD26 immunotoxins.

Selective killing of resting PBMC by bispecific immunotoxin. The results of the functional assays above suggested that the bispecific immunotoxin may be more effective against activated cells than against resting cells and additionally suggested that its action might be limited to the CD4⁺CD26⁺ cells that provide help for Ig synthesis, because the efficacy at inhibiting a polyclonal PHA response was low. To assess actual specificity directly, we incubated resting and PHA-activated T cells with the immunotoxins and determined the phenotypes of the cells surviving after 4 days (Fig 8). The PHA activation was chosen because this gave the clearest difference in the functional assays described above. In this experiment, using either resting or PHA-activated T cells, the anti-CD26 immunotoxin was not effective.
against any of the cell phenotypes determined. Against resting cells, the anti-CD4–bRicin conjugate yielded an a 50% effective dose (ED$_{50}$) of $10^{-10}$ against all CD4$^+$ cells, which was unrelated to the expression or lack of CD26. The bispecific immunotoxin was as ineffective as the anti-CD26–bRicin. On examining the efficacy against PHA-activated cells, a different picture emerged. First, the toxicity of the anti-CD4 immunotoxin fell by an order of magnitude, and this correlated with a decrease in surface expression of CD4 measured by immunofluorescence (mean cell fluorescence fell from 10.82 to 6.69). The expression of CD26 increased on the blast cells (mean cell fluorescence rose from 2.823 to 4.464), and this correlated with an increase in the efficiency of the bispecific antibody to eliminate CD4$^+$CD26$^-$ cells. A large difference remained between the anti-CD4–bRicin and the bispecific immunotoxin in their capacities to eliminate CD4$^+$CD26$^-$ cells (20-fold at the highest concentration), but the gap was significantly reduced against CD4$^+$CD26$^+$ cells (4-fold at the highest concentration). The addition of lactose (40 mmol/L) to the incubations had a minimal effect on the toxicity of the bispecific immunotoxin in this cell survival assay reducing the efficacy by a factor of 2 against CD4$^+$CD26$^+$ cells (data not shown).

Elimination of the alloantigenic response by bispecific immunotoxin. In Fig 9, the results are shown for the effect of the parental and bispecific immunotoxins on the induction of a HMLR. In this assay, the bispecific immunotoxin proved to be twofold to threefold more toxic than the anti-CD4 immunotoxin and twofold more efficacious than the anti-CD26 immunotoxin. The bRicin control was ineffective at preventing the HMLR.

**DISCUSSION**

In this report, we describe the use of a bispecific antibody to target activated T cells through the simultaneous expression of CD4 and the activation antigen, CD26, which has more recently been shown to be DPP IV.$^{13,14}$ In pursuit of our aim to design agents that may modulate GvHD, we set out to establish an immunotoxin that exerted its specificity through its ability to bind two T-cell antigens and developed
Fig 7. Efficacy of immunotoxins to eliminate the ability of resting and preactivated CD4+ T cells to provide help for PWM-induced B-cell IgG synthesis. All assays were performed in triplicate and the SEM was less than 15% of the mean. Note that the ordinate is a logarithmic scale.

its cytotoxicity through its ability to be internalized and release its toxin only after bivalent binding. Other groups have attempted to target activation antigens on T cells, aiming in particular at the interleukin 2 (IL-2) receptor, and the results have been variable.\textsuperscript{26} Additionally, CD4 has been considered a strong candidate for targeting, and anti-CD4 antibodies, either unconjugated or as immunotoxins, have been shown to generate powerful immunosuppressions.\textsuperscript{10} In fact, throughout our own results presented here, the anti-CD4-bRicin developed the strongest cytotoxicity against resting T cells in the majority of our assays. However, an anti-CD4 immunotoxin may be too strong in its potential in that it removes T cells that are not activated and are thus not contributing to any antagonistic immune response, and secondly, it may remove a very important population of CD4+CD45RA+ suppressor-inducer cells\textsuperscript{30} which may help downregulate an antagonistic response. For these reasons, we chose to try and limit the toxicity of an anti-CD4 by introducing a specificity for the T-cell activation marker CD26 in the context of a bispecific antibody.

We prepared a bispecific hybrid antibody, which constituted 31\% of the total hybrid heavy chain material secreted by the quadroma. This is in agreement with the 25\% that would be expected if random association of heavy and light chains occurs.\textsuperscript{28} Having prepared a hybrid antibody with demonstrable bispecificity, we clearly show that monovalent binding of CD4 or CD26 does not result in significant modulation (assessed by fluorescence and radiolabeling) but that the bispecific antibody, monovalent for each specificity, does retain the ability to modulate. This modulation was shown to be the result of internalization and not shedding, thus allowing a mechanism for introduction of the toxin moiety into the interior of the cell. It is now apparent that much of the efficacy of a targeted toxin may not be related to the initial binding affinity, which is nevertheless important, but rather to the fate of the toxin-cell ligand complex on internalization. For antibody-mediated internalization, two paths may be followed. The first path involves endocytosis followed by recycling of the receptor and antibody to the cell surface, whereas the second path involves entry into the lysosomal compartment followed by degradation.\textsuperscript{29} Bivalent binding tends to result in the antibody complex entering the lysosomal pathway, entry into the cell is quick, but this is offset by rapid degradation. Univalent binding favors the endocytic pathway that results in slow entry into the cell, but degradation is also slower. However, in either case, antibody-delivered toxins seem to be equally effective. Recent results suggest that internalized CD4 enters the more rapid lysosomal degradation pathway.\textsuperscript{30} For CD26, its slow reexpression (6 days) after modulation implies de novo synthesis rather than recycling suggesting that internalized CD26 also enters the lysosomal degradation pathway.\textsuperscript{19} It is interesting to contemplate which pathway would dominate entry mediated by the bispecific antibody if CD4 and CD26 differed in their intracellular routes.

It is important to note that the modulation mediated by the bispecific antibody was minimal on resting E+ cells but
Fig 8. Estimate of the survival of T-cell subsets after a 4-day incubation with immunotoxins. Resting E+ cells and PHA-activated blasts were incubated with anti-CD26-bRicin, anti-CD4-bRicin, or bispecific anti-CD4-anti-CD26-bRicin. After 4 days, viable cells were isolated by centrifugation over Ficoll-Hypaque, and the absolute numbers of CD4+CD26-, CD4-CD26+, and CD4+CD26+ cells were determined. Note that the ordinate is a logarithmic scale.

Fig 9. Effect of the immunotoxins on the HMLR. The immunotoxins or bRicin control were added to the HMLR for the full 6 days of the reaction at the concentrations indicated. The vertical bars indicate the standard error of the mean. The HMLR response in the absence of any addition was 21,246 ± 2,209 and the response against autologous stimulators was 3,211 ± 1,102.
of CD26+ cells remained high according to the phenotypes of the survival experiment, it is possible that the anti-CD26 was blocking here rather than killing. This is of consequence, because it is clear that a large proportion of bispecific antibody binds, probably monovalently, but is not internalized. This is apparent in the modulation experiments where on binding of the bispecific antibody, the same proportion of cells remained positive after modulation, but the antigen density was very much reduced. However, the antibody remaining unmodulated on the surface would still be blocking any residual CD4 and CD26 (all incubations were performed under saturating antibody conditions) and essentially inducing the anergic state in any cell not killed by internalization of the toxin. However, in real terms, our concerns may not be relevant. The clear advantage of the immunotoxin is that it generates an efficient kill at concentrations in the $10^{-10}$ mol/L range; meanwhile, the effective blocking of surface CD26 mentioned above required an antibody concentration at least 50-fold higher.\textsuperscript{12}

We have evidence (not presented here) that at $10^{-10}$ mol/L concentrations, the bispecific immunotoxin spares both CD8\textsuperscript{+} cells and CD4\textsuperscript{+}CD45RA\textsuperscript{+} cells, with the former leading to the cytotoxic cells generated in a HMLR. Nevertheless, the ability of the bispecific immunotoxin to reduce the HMLR to baseline implies that the proliferation of the CD8\textsuperscript{+} cells is completely dependent on help provided by the activated CD4\textsuperscript{+}CD26\textsuperscript{+} cells. This is not the case for the PHA responses where the baseline bottomed out at 20\% of the maximal response implying survival of a non-CD4\textsuperscript{+} population, which is presumably CD8\textsuperscript{+}. In the response to PHA, of course, CD8\textsuperscript{+} cells are less dependent on the help provided by CD4\textsuperscript{+} cells. This has important implications for GvHD where both CD4\textsuperscript{+} and CD8\textsuperscript{+} cells are involved in the pathologic process.

Accordingly, in this report we describe our successful attempts to target activated T cells. The bispecific antibody we use is minimally effective against resting T cells, but it performs well in eliminating CD4\textsuperscript{+}CD26\textsuperscript{+}--activated T cells. In contrast, the anti-CD4 immunotoxin is highly toxic against resting T cells, but its efficacy falls by an order of magnitude against activated T cells. It is apparent that bispecific antibodies may thus be used not only to bridge effector cells to target cells, as has been described in many reports,\textsuperscript{5,15} but also to bridge antigens on the same cell and to deliver a toxin moiety to the cell interior. Our experience shows that the efficacy of such bispecific immunotoxins is completely dependent on the nature of the antigens targeted. For example, we have preliminary results that indicate that a CD4\textsuperscript{+}CD45RA\textsuperscript{+} bispecific antibody is not internalized, and that this lack of modulation can be attributed to the CD45RA antigen. Consequently, as our understanding of the dynamic properties of proteins of the T-cell surface increases, we may be able to use such knowledge to further develop bispecific immunotoxins, such as the anti-CD4--anti-CD26-bRicin described here, which may exhibit fine specificity in exerting their cytotoxic potential, and consequently help prevent GvHD and autoimmune processes in vivo.
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