Characterization of Novel Costimulatory Molecules

A PROTEIN OF 38–42 kDa FROM B CELL SURFACE IS CONCERNED WITH T CELL ACTIVATION AND DIFFERENTIATION*

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Optimal activation of T cells often requires signals delivered by the ligation of T cell receptor (TCR) and those resulting from costimulatory interaction between certain T cell surface accessory molecules and their respective counter receptors on antigen presenting cells. The molecular events underlying the co-stimulatory activity are still not understood fully. Here we describe a 38–42-kDa (B3) protein, present on the surface of lipopolysaccharide-activated B cells, which can provide costimulation to resting T cells leading to a predominant B cell activity complex restricted (1), and the second non-major histocompatibility complex restricted (1), and the second non-major histocompatibility complex restricted signal (costimulatory signal) is delivered by certain molecules present on the surface of APCs (4–6). The participation of costimulatory signal in T cell activation is of paramount importance as it results in two potential outcomes, activation or clonal anergy (7, 8). The two different outcomes of antigen recognition, by T cells, is first explained by the dual signal model of T cell activation by Bretscher and Cohn (9) and updated recently by Jenks and Schwartz (10). Since then, efforts of numerous researchers have culminated in the identification of various molecules capable of providing costimulatory signal (11). The list of these second signal generating molecules, however, is still far from complete as reports are rapidly appearing in the literature regarding the possible existence of certain hitherto unknown molecules with costimulatory properties (12–40).

To identify additional cell surface-associated molecules that provide costimulatory signals to T cells, we have isolated proteins from lipopolysaccharide (LPS)-activated B cell membrane. When reconstituted into lipid bilayer, at least three proteins (B1, B2, and B3) gave differential levels of costimulatory help to primary T cell activation. The present document presents the results obtained with one of the above proteins with a molecular mass range of 38–42 kDa (B3) (the data of B1 and B2 have been communicated elsewhere) and its relation to primary T cell activation and differentiation.

EXPERIMENTAL PROCEDURES

Animals
Female inbred BALB/C mice, 8–10 weeks old, were obtained from the National Institute of Nutrition, Hyderabad, India, and from our conventional conditions and were allowed free access to food and water.

Cell Lines and Hybridomas Used
All the cell lines and hybridomas used in this study, viz. anti-Thy 1 (TIB 99), anti-L3T4 (TIB 207), anti-CD8 (TIB 150), anti-Mac2 (TIB 166), anti-Mac3 (TIB 168), 33D1 (anti-dendritic cell Ab; TIB 227), anti-IL-2 R (CRL 1698), HT-2 (CRL 1841), anti-IFN-γ (HB 170), anti-IL-2 (HB 6794), anti-IL-4 (HB 188), anti-LFA-1 (TIB 217), and anti-ICAM-1 (CRL 1878) were procured from the American Type Culture Collection (ATCC), Rockville, MD. Anti-CD3 (145.2C11) was a kind gift from Dr. Charles A. Janeway Jr., Howard Hughes Medical Institute, New Haven, CT. WEHI-279 (CRL 1704), A20 (TIB 208), and anti-HSA (TIB 183) were kind gifts from Dr. Satyajit Rath, National Institute of Immunology, New Delhi, India.

Primary T Cells

CD4+ T cells were prepared from mice spleens as follows. Briefly, a single cell suspension of spleens was prepared in balanced salt solution (pH 7.2). Red cells were lysed using hemolytic Gey’s solution. Non-adherent cells, collected by allowing cells to adhere to plastic Petri plates (Nunc, Denmark) at 37 °C with 7% CO2 for 2 h, were treated sequentially with a mixture of anti-Mac2 and anti-Mac3 (45 min on ice), 33D1 (45 min on ice), and anti-IL-4 (45 min on ice). The cells were then washed and incubated with two rounds of anti-Lyt-2.2 (Cedarlane, Ontario, Canada) with 45 min each on ice followed by treatment with low toxicity baby rabbit complement. CD4+ T cells were enriched by passing through nylon wool column. The cells were collected after five to six washes with prewarmed RPMI, 10% FCS (37 °C) and plated on Petri plates, previously coated with goat anti-mouse IgM, for 1 h at 37 °C. The non-adherent cells were used as a source of CD4− T cells and the purity of such cell population routinely exceeded 98% as estimated by FACScan (Beckton Dickinson) in preparations stained with anti-L3T4.

T helper cell activation is accomplished by recognition of antigen-Ia complex, expressed by antigen presenting cell (APC), via a donally restricted heterodimeric receptor (TcR) (1–3). The precise mechanism by which APCs activate T cells is quite complex and not fully understood. The current dogma is that at least two signals are required. The first signal is provided by the occupancy of TcR, which is major histocompatibility complex restricted (1), and the second non-major histocompatibility complex restricted signal (costimulatory signal) is delivered by certain molecules present on the surface of APCs (4–6). The participation of costimulatory signal in T cell activation is of paramount importance as it results in two potential outcomes, activation or clonal anergy (7, 8). The two different outcomes of antigen recognition, by T cells, is first explained by the dual signal model of T cell activation by Bretscher and Cohn (9) and updated recently by Jenks and Schwartz (10). Since then, efforts of numerous researchers have culminated in the identification of various molecules capable of providing costimulatory signal (11). The list of these second signal generating molecules, however, is still far from complete as reports are rapidly appearing in the literature regarding the possible existence of certain hitherto unknown molecules with costimulatory properties (12–40).

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Preparation of Resting B Lymphocytes

Resting B cells from mice spleens were prepared as follows. Briefly, a single cell suspension of spleens was prepared in balanced salt solution (pH 7.2). The red blood cells were removed by treatment with Gey's solution. After removing the macrophages by allowing them to adhere twice to plastic surface (1 h each at 37 °C and 7% CO2), the cells were treated twice (45 min each on ice) with a mixture of anti-Mac-2, anti-Mac-3, and 3D1 and a mixture of anti-Thy1, anti-L3T4, and anti-CD8 followed by labeling (30 min at 37 °C) with low toxicity baby rabbit complement. The cells obtained were then loaded on a discontinuous Percoll gradient (100, 70, and 50%) and centrifuged at 1600 × g for 30 min at 4 °C. The cells collected from 100–70% interface layer were considered as resting B cells.

Preparation of LPS-activated B Lymphocytes

Activated B cells were prepared as follows. Briefly, a single cell suspension of mice spleens was prepared in balanced salt solution (pH 7.2). The red blood cell were depleted by treatment with hemolytic Gey's solution. The cells were then plated on plastic Petri plates (Nunc, Denmark) for 2 h at 37 °C and 7% CO2. The non-adherent cells were collected via centrifugation at 45 min each with a mixture of anti-Mac-2 and anti-Mac-3, and a mixture containing anti-Thy1, anti-L3T4, and anti-CD8 antibodies followed by complement mediated killing. The cells were then incubated at a concentration of 4 × 10^7 ml/10 ml per plate with 10 μg/ml LPS (from Salmonella typhosa; Sigma) for 72 h at 37 °C and 7% CO2. The purity of such cells was over 98% as analyzed by FACSscan (Beckton Dickinson).

Isolation of LPS-activated B Cell Surface Proteins

The LPS-activated B cells were harvested and washed three times with PBS (pH 7.2) and frozen overnight at −70 °C. The cells were thawed and homogenized in the presence of 0.25 M sucrose, 20 mM Tris-HCl (pH 7.4), and 1 mM EDTA, and 100 mM NH4HCO3, 50 mM Tris-HCl, 0.1 mM EDTA, and 0.15 M NaCl (pH 7.2). The red blood cell were depleted by treatment with hemeolytic physiological saline and later passed through a Sephadex G-50 mini-column according to Fry et al. (17) to remove unliposomized protein.

Density Gradient Centrifugation—2 ml of the above sample was layered on top of a discontinuous gradient of 5-30% glycerol (w/v) in 10 mM Tris-HCl (pH 6.8), 0.15 mM NaCl, and 0.1 mM EDTA. The sample was centrifuged overnight at 98,000 × g in a Beckman SW28 rotor at 4 °C. The pellet was dissolved in physiological saline and stored sterile for more than 3 months at −20 °C. The protein content (w/v) in liposomes, was estimated after lysing with 1% SDS by the BCA method (18). The lipid phosphorous was estimated as per the method of Ames and Dubin (19).

T Cell Proliferation Assay—CD4+ T cells were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with penicillin (70 μg/ml), streptomycin (100 μg/ml), glutamine (4 mM), 2-mercaptoethanol (50 μM), sodium pyruvate (1 mM), HEPES (20 μM), and 10% heat-inactivated FCS (Sera Laboratories, Crawley Down, Sussex, United Kingdom). Affinity purified anti-CD3 (145.2C11) (10 μg/ml), diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6), was immobilized on the surface of 96-well flat-bottomed (Costar, Cambridge, MA) culture plates by overnight incubation at 4 °C. The wells were washed three times with PBS before adding the cells along with varying concentrations of experimental, reconstituted, and control proteins. Phorbol myristate acetate (PMA) (Sigma) was used at a concentration of 10 ng/ml. The proliferation was assessed after 72 h of culture with 1 μCi (H)thymidine added during the last 16 h of culture. Cells were harvested on a multiple sample harvester (Skatron, Norway), and the incorporated radioactivity was assessed in a scintillation counter.

Lymphokine Bioassay—CD4+ T cells were cultured in 24-well plates at a density of 0.25 × 10^6/well with different stimuli. The culture supernatants were collected after 22 h for lymphokines assay.

Interleukin-2 and -4 Assay—Interleukin-2 and interleukin-4, in culture supernatants, were determined by the induction of HT-2 proliferating cells described by Fernandez-Bortan et al. (2). Briefly, HT-2 cells (1 × 10^5/well) were cultured in 96-well flat bottomed plates, containing RPMI 10% FCS and various concentrations of culture supernatants from control and experimental wells. Since HT-2 cells are responsive to both these lymphokines, therefore, while measuring IL-2 level, the activity of IL-4 was neutralized using anti-murine IL-4 antibody (600 ng/ml). Likewise, for IL-4 assay, the activity of IL-2 was inhibited by adding anti-IL-2 and anti-IL-2 receptor antibodies. The cultures were incubated for 24 h at 37 °C/7% CO2 followed by pulsing with 1 μCi (H)thymidine during the last 6 h of culture. The cells were harvested, and the incorporated radioactivity was measured by liquid scintillation counting. The lymphokines activity, in terms of units, was derived by extrapolating the standard curve values obtained by using recombinant interleukin-2 and -4 (Genzyme).

Interferon-γ Assay—Interferon-γ was assayed by its ability to inhibit the proliferation of WEHI-279 cells. Cells were cultured in 96-well flat bottomed plates with different concentrations of culture supernatants from control as well as experimental wells. The cultures were pulsed with (H)thymidine and the incorporated radioactivity was measured as above. Recombinant IFN-γ served as standard to extrapolate the lymphokine activity in terms of units.

Interleukin-5 Assay—The activity of IL-5 was assayed by the induction of proliferation of mouse splenic resting B cells using dextran sulfate as a comitogen as described by Swain (21). Resting B cells were obtained from splenocyte suspension as mentioned above. After washing, 10^6 cells/well were cultured in 96-well flat bottomed microtiter plate. 100 μl aliquots of a range of dilutions of the culture supernatants under test were added in triplate wells along with 50 μg/ml dextran sulfate. Murine recombinant IL-5 added in different dilutions to obtain a standard curve whose specificity was cross-checked with anti-IL-5 (500 ng/ml). The cultures were pulsed with 1 μCi (H)thymidine and harvested 3 days later. The incorporated radioactivity was determined by liquid scintillation counting. IL-5 activity, expressed in terms of units/ml, was obtained from the standard values.

Northern Blotting of mRNA—CD4+ T cells (5 × 10^6/ml) were cultured in 24-well plate (Costar, MA) for 8 h at 37 °C, 7% CO2 in the presence of previously immobilized anti-CD3 (10 μg/ml), PMA (10 ng/ ml), and 0.1 μg/ml (0.1 μCi/ml) in 20% FCS. After 8 h, the cells were harvested and washed repeatedly in cold PBS (pH 7.2) and stored at −70 °C in pellets until RNA extraction was performed.
Total cytoplasmic RNA was prepared according to White and Bancroft (22) and blotted over to Immobilon-N (Millipore, MA) and cross-linked to the membranes by Stratallinker 1800 (Stratagene, La Jolla, CA). Membranes were washed with 1 × SSC and 0.1% SDS for 1 h at 65°C and then prehybridized in a solution containing 50% formamide, 5 × SSPE (20 × SSPE = 3 × NaCl, 0.2 M Na2HPO4, and 20 mM EDTA) and 5 × Denhardt’s reagents. Hybridization was performed at 1% each of Formamide, Back fraction V, polyvinylpyrrolidone, 0.1% SDS) overnight at 42°C. Specific RNA was detected by probing the membranes with [γ-32P]ATP-labeled cDNA probes for murine IL-2, IL-4, IL-5, and IFN-γ (Amgen Biological, Thousand Oaks, CA) in fresh prehybridization buffer for 24 h at 42°C. After washing, the membranes were exposed to x-ray film with an intensifying screen at −70°C for 48 h.

**Electron Microscopy**

Negative Staining of Liposomes—A 20-μl droplet of aqueous suspension of liposomes was placed on a fresh piece of paraffin, and a sample droplet was picked up by touching a carbon-coated grid to it. After allowing the excess liquid to drain off, the grid was gently dipped in 1% phosphotungstic acid (pH 7.0) and dried by blotting on a filter paper. Grids were observed in a transmission electron microscope (J EOL 1200 EX II, Japan) and representative fields were photographed.

**Western Blotting**—Western immunoblots were made from SDS-PAGE. The reagents for this purpose were purchased from Amersham (Arlington Heights, IL) and used as described above, with purification by SDS-PAGE (23). The gel was stained with Coomassie Blue, destained, and its ability to bind 125I-ConA was tested in conjunction with autoradiography on x-ray film.

**Electrophoretic and Lectin Gel Binding Analysis**—SDS-PAGE was performed in 0.5-mm thick slab gels containing 4% acrylamide in stacking gel and a 10% acrylamide in separating gel, with a buffer system according to Laemmli (15). The lane containing B3 was transferred onto Immobilon-P (Millipore, CA), washed, and visualized by staining with 0.2% aqueous Ponceau S, destained, and autoradiography on x-ray film.

**Western Immunoblotting**—Western immunoblots were made from SDS-PAGE analysis of LPS-activated B cell membrane, stained with Coomassie blue, revealing protein bands. a, the sample was analyzed on a 10% gel under non-reducing conditions. The arrowhead denotes the position of B3, b and c, one-dimensional profile of B3 under non-reducing (b) and reducing (c) conditions (see arrowheads). d, SDS-PAGE analysis of resting B cell membrane. The sample was run on 10% gel under non-reducing conditions and probed with the same antibody. The arrowhead denotes the possible position of B3. e, two-dimensional non-reducing (SDS-PAGE)/reducing (TDAB-PAGE) electrophoretic pattern of B3. Gel electrophoresis was performed on 10% gels in both the dimensions and stained with Coomassie Blue. f, lectin gel binding analysis of B3. This was performed with 125I-ConA as outlined under “Experimental Procedures.” The arrowhead indicates the position of B3. g, phosphorylation assay of B3. The B cell membrane proteins were subjected to phosphorylation analysis with [γ-32P]ATP. B3 was located, isolated, by PAGE, and analyzed by autoradiography.

**Protein Sequencing**—Protein sequencing of the 38–42-kDa B Cell Surface Protein and T Cell Stimulation was carried out using a Procise Sequencer (model 492A) at The Protein Sequencing Facility, Worcester Foundation for Experimental Biology, Shrewsbury, MA.

**F I G . 1** SDS-PAGE analysis of LPS-activated B cell membrane, stained with Coomassie blue, revealing protein bands. a, the sample was analyzed on a 10% gel under non-reducing conditions. The arrowhead denotes the position of B3, b and c, one-dimensional profile of B3 under non-reducing (b) and reducing (c) conditions (see arrowheads). d, SDS-PAGE analysis of resting B cell membrane. The sample was run on 10% gel under non-reducing conditions and probed with the same antibody. The arrowhead denotes the possible position of B3. e, two-dimensional non-reducing (SDS-PAGE)/reducing (TDAB-PAGE) electrophoretic pattern of B3. Gel electrophoresis was performed on 10% gels in both the dimensions and stained with Coomassie Blue. a, the sample was analyzed on a 10% gel under non-reducing conditions. The arrowhead denotes the position of B3, b and c, one-dimensional profile of B3 under non-reducing (b) and reducing (c) conditions (see arrowheads). d, SDS-PAGE analysis of resting B cell membrane. The sample was run on 10% gel under non-reducing conditions and probed with the same antibody. The arrowhead denotes the possible position of B3. e, two-dimensional non-reducing (SDS-PAGE)/reducing (TDAB-PAGE) electrophoretic pattern of B3. Gel electrophoresis was performed on 10% gels in both the dimensions and stained with Coomassie Blue. f, lectin gel binding analysis of B3. This was performed with 125I-ConA as outlined under “Experimental Procedures.” The arrowhead indicates the position of B3. g, phosphorylation assay of B3. The B cell membrane proteins were subjected to phosphorylation analysis with [γ-32P]ATP. B3 was located, isolated, by PAGE, and analyzed by autoradiography.
RESULTS

Identification and Partial Characterization of B3—The membranes of LPS-activated murine splenic B cells, when subjected to SDS-PAGE analysis, revealed about 15 major protein bands when stained with Coomassie Brilliant Blue (Fig. 1a). B3 was localized, crushed, and eluted as described under “Experimental Procedures.” The accuracy with which B3 was isolated was checked by rerunning the protein on SDS-PAGE. When stained with Coomassie Brilliant Blue, it demonstrated a single band both under non-reducing (Fig. 1b), reducing (Fig. 1c), and as a single spot in two-dimensional gel electrophoretic (Fig. 1e) conditions. When B3 was subjected to lectin gel binding (Fig. 1f) and phosphorylation (Fig. 1g) assays it was noticed that B3 binds 125I-ConA and incorporates radiolabeled phosphate indicating the possibility that it is a phosphoglycoprotein. The results of our attempts to localize B3 on the surface of resting B cells demonstrated that it is hardly detectable even when probed by silver stain of SDS-gel possibly indicating that its high expression is induced when pretreated with LPS (Fig. 1d).

B3 Binds to T Cell Surface—B3, before checking for its co-stimulatory ability, was reconstituted into lipid bilayers. Prior to reconstituting the protein, it was ensured that a fairly homogeneous preparation of liposomes was obtained. When stained with Coomassie Brilliant Blue, it demonstrated a single band both under non-reducing (Fig. 1b), reducing (Fig. 1c), and as a single spot in two-dimensional gel electrophoretic (Fig. 1e) conditions. When B3 was subjected to lectin gel binding (Fig. 1f) and phosphorylation (Fig. 1g) assays it was noticed that B3 binds 125I-ConA and incorporates radiolabeled phosphate indicating the possibility that it is a phosphoglycoprotein. The results of our attempts to localize B3 on the surface of resting B cells demonstrated that it is hardly detectable even when probed by silver stain of SDS-gel possibly indicating that its high expression is induced when pretreated with LPS (Fig. 1d).

Fig. 2. Binding and distribution of B3 on T cell surface. B3 coupled to liposomes was labeled with 125I and incubated with anti-CD3-activated CD4+ T cells and EM autoradiography was performed as described under “Experimental Procedures.” The cells were examined by transmission electron microscopy. a, negative staining of liposomes by phosphotungstic acid (×10,000, bar = 200 nm). b, 125I-labeled B3 coupled to liposomes. Arrows show the liposomes frequently bearing the iodinated B3 (×20,000, bar = 200 nm). c, CD4+ T cells labeled with iodinated B3 (arrows; ×20,000; bar = 200 nm). Note the distribution of B3 only on the T cell surface.

To further confirm the specificity of binding of B3 to T cells, competitive binding assay was performed. When unreconstituted 125I-B3 was incubated with T cells in the absence of anti-CD3, very less binding (1,250 ± 252 cpm) was noticed. However, the number of receptors for B3 appeared to be up-regulated when the T cells were preactivated with anti-CD3.
the half-maximal concentration (0.01 M) was observed. Thus, in all the subsequent experiments, only this concentration of B3 was increased, no further amplification in T cell proliferation with cells plus anti-CD3 only. When the concentration of B3 in [3H]TdR incorporation of T cells in a dose-dependent manner showed that the addition of B3 to the cultures led to an increase of 10,509 ± 2,827 cpm (Fig. 6a, f). This was further confirmed by Northern analysis, the details of which are highlighted in Fig. 7 (see inset).

Reverse Phase-HPLC and Protein Sequence Analysis—B3, when subjected to reverse phase-HPLC, showed a single prominent peak (at 18.21 min) in the chromatogram (Fig. 8a) demonstrating thereby that this protein is homogeneous in its present form of isolation coinciding with our two-dimensional data. This protein was subjected to internal amino acid sequence upon tryptic digestion (because of its blocked N terminus) up to 15 residues, and the details are depicted in Fig. 8b.

**DISCUSSION**

Optimal T cell activation depends not only on the occupancy of TcR, but also on accessory molecules, provided by the APCs, that function in cell-cell adhesion and/or signal transduction (26). During the recent past, several new costimulatory molecules have been identified (12–14), and the list still appears to be incomplete as the evidence for existence of additional cell surface proteins involved in signal transduction is being raised by several workers (27, 28). It may be mentioned here that on the basis of their ability to secrete specific lymphokines, T helper cells have been divided into Th1 and Th2. Th1 cells produce IL-2 and IFN-γ, lymphotoxin, etc., and primarily participate in stimulating the cell-mediated immunity (29, 30), while Th2 cells secrete IL-4, IL-5, IL-6, etc., and are involved mainly in the induction of humoral immunity (29, 31–34). It has been postulated that these two T helper subsets are not only functionally different but also need qualitatively and quantitatively distinct requirements for costimulation (35). However, to date and to the best of our knowledge, no costimulatory molecules are reported which exclusively activate Th2 cells.

In the present study, we describe the biochemical and functional analysis of a novel LPS-activated murine splenic B lymphocyte cell surface-associated costimulatory molecule, provi-

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**Fig. 4. Competitive binding assay with B3.** CD4+ T cells (1 × 10^6/ml) were incubated with or without anti-CD3 or unlabeled B3 30 min at 37°C. After washing, [125I]-labeled B3 (2 ng) was added to final a volume of 200 μl and incubated for 2 h at 4°C with gentle agitation. After extensive washing, the incorporated radioactivity in cell pellets was monitored on a gamma counter. a, cells + [125I]-B3; b, cells + anti-CD3 + [125I]-B3; c, cells + anti-CD3 + 100 μg/ml B3; d, cells + anti-CD3 + 25 μg/ml B3; e, cells + anti-CD3 + 5 μg/ml B3; f, cells + anti-CD3 + 2 μg/ml B3; g, cells + anti-CD3 + 1 μg/ml B3. The range of dilutions of unlabeled B3 used was in terms of protein content.

**Fig. 5. Western blotting.** Proteins were run on SDS-PAGE (10%) and transferred onto Immobilon-P membrane and stained in 0.2% aqueous Ponceau S to ensure that all lanes contained the transferred protein. After washing and blocking, the membrane was probed with antibodies against ICAM-1, LFA-1α, HSA, B7, and VCAM-1. As a positive control, LPS-activated B cell membrane sample was run in appropriate lanes, and the bound antibody was detected by diaminobenzidine.

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(10,509 ± 2,562 cpm). Further, the binding specificity of B3 to T cells was tested by competing [125I]-B3 with unreconstituted and unlabeled B3 to bind the anti-CD3-activated T cells. The data clearly show that the binding capacity of [125I]-B3 is diminished by unlabeled B3 (Fig. 4, a-g).

Antibodies against Murine LFA-1α, ICAM-1, HSA-1, B7, and VCAM-1 Do Not Cross-react with B3—It is known that a majority of costimulatory molecules identified thus far are adhesive in nature. In our efforts to rule out the possibility of B3 being a known costimulatory molecule, a Western analysis was performed. The data obtained are depicted in Fig. 5 which demonstrates that B3 did not cross-react with any of the antibodies against murine LFA-1α, ICAM-1, HSA-1, B7, and VCAM-1. LPS-activated B cell membrane lysate was run in appropriate lanes as a positive control for these adhesive molecules.

B3 Costimulates Primary T Cells to Proliferate—Fig. 6a shows that the addition of B3 to the cultures led to an increase in [3H]TdR incorporation of T cells in a dose-dependent manner. A maximum statistically significant (p < 0.05) proliferation (39,426 ± 2,144 cpm) was noticed at a B3 concentration of 1 μg/ml as against the basal value (955 ± 268 cpm) obtained with cells plus anti-CD3 only. When the concentration of B3 was increased, no further amplification in T cell proliferation was observed. Thus, in all the subsequent experiments, only the half-maximal concentration (0.01 μg/ml) of B3, as determined by dose-response pattern, was used. Further, when the cultures were stimulated with controls like liposomes, SDS, B3 alone, gel eluate, and LPS, no statistically significant (p < 0.05) proliferation (~2,000 cpm) of T cells was noticed (Fig. 6b) pointing out thereby that the activity elicited by B3 was indeed specific. On the other hand, when PMA was added to anti-CD3-

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**Fig. 5. Western blotting.** Proteins were run on SDS-PAGE (10%) and transferred onto Immobilon-P membrane and stained in 0.2% aqueous Ponceau S to ensure that all lanes contained the transferred protein. After washing and blocking, the membrane was probed with antibodies against ICAM-1, LFA-1α, HSA, B7, and VCAM-1. As a positive control, LPS-activated B cell membrane sample was run in appropriate lanes, and the bound antibody was detected by diaminobenzidine.
sionally termed B3, that chiefly activates Th2-like cells. Our data suggest that B3 molecule is specifically involved in the costimulation of resting T helper cells upon cross-linking TcR-CD3 complex with anti-CD3 monoclonal antibody resulting in predominant secretion of IL-4 and IL-5 and very poor levels of IL-2 and IFN-γ. Our rationale for choosing B cell surface molecules to provide costimulatory signal to T cells lies in the observation that B lymphocytes are major APCs for the clonal expansion of normal murine CD4⁺ T cells (36). Further, our selection of LPS-activated B cells for identifying the costimulatory molecules is based on the premise that resting B cells are poor APCs (37) and do not constitute costimulatory activity (38, 39); only upon treatment with either LPS or IL-1 or immunoglobulin or IFN-γ, or cross-linking surface major histocompatibility complex class-II molecules or neuraminidase etc. (36, 40–42) do the B cells acquire enhanced ability to stimulate T cells. Moreover, cytokine secretion is induced from naïve T cells only when activated B cells are used as APCs (43). It may also be stressed here that the molecule, described in the present study, is barely detectable on the membranes of resting B cells even when loaded five times the concentration of LPS-activated B cell membrane lysate probed with silver strain.

In biochemical experiments, we have characterized B3 as a single molecule with an approximate molecular mass of 38–42 kDa when analyzed by SDS-PAGE. Upon reducing, this molecule was recovered as a single sharp band. The reverse phase-HPLC approach to purify this protein clearly showed a solitary and distinct peak (at 18.21 min) in the chromatogram. This information, in conjunction with the SDS-PAGE analysis, conclusively proves that this molecule is homogeneous in its present form of isolation. In addition to these approaches, the two-dimensional profile of B3 always consistently yielded a single pattern (in about 12 repetitions) which also reiterates the absence of any other contaminants sticking either specifically or nonspecifically to the said protein. As assessed by its ability to bind ¹²⁵I-ConA, B3 appears to be glycosylated a fact which was further substantiated by partial digestion of this protein by N-glycosidase F that resulted in two distinct fragments of 22 and 18 kDa (data not shown). The phosphorylation assay, on the other hand, revealed that this molecule is capable of incorporating radiolabeled phosphate. These results indicate that B3 is a phosphoglycoprotein.

For an effective signal transduction, a costimulatory molecule is expected to bind its counter ligand on the target cell. Studies were undertaken to explore this possibility, and the results obtained indicate that B3 molecule binds to T cells and this binding can be diminished by competing with unlabeled B3. This fact is further strengthened by the results obtained with electron microscopic autoradiographic studies which show that ¹²⁵I-labeled reconstituted B3 molecule when incubated with anti-CD3-activated T cells, this protein is found associ-

![Image](image-url)
The only known costimulatory molecule closest to B3, in terms of molecular weight, is B7–2. Like B7 (now called CD80), B7–2 (a 34-kDa protein) is a counterreceptor for CD28 and CTLA-4. T cell surface molecules and induces the predominant secretion of IL-2 (51). In contrast, the molecule described in the present study activates CD4+ Th cells to secrete IL-4 and IL-5 but a very little IL-2 and IFN-γ. These observations lend support to the view that B3 molecule is not a counter ligand for either CD28 or CTLA-4. Also, when anti-CD3-activated T cells incubated with anti-CD28 and were allowed to interact with 125I-labeled B3, there was no substantial change in the binding capacity of labeled B3 to T cells. Further on, our experiments to identify the receptor for B3 on T cells, using the homobifunctional cross-linker disuccinimimidyl suberate, clearly demonstrated B3 binds a 60-kDa protein on T cell surface (data not shown). It may be mentioned here that B7 (CD80) binds a 44-kDa glycoprotein (CD28) on T cell surface.

Thus, all the generated evidence favors the conclusion that B3 is a novel costimulatory molecule that activates Th2-like cells. Using a similar approach, we have recently demonstrated the presence of a 150-kDa protein (M150) from the membranes of thioglycollate-elicited murine peritoneal macrophages that selectively activate Th1 type of cells leading to the secretion of significant levels of IL-2 and IFN-γ but negligible amounts of IL-4 (52).

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