

MONOCLONAL ANTIBODIES SPECIFIC FOR INTERLEUKIN 3-SENSITIVE MURINE CELLS

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Interleukin 3 is a 26–28 kD glycoprotein produced by antigen- and lectin-stimulated T lymphocytes, and constitutively released in culture by the mouse tumor cell line WEHI-3 (1). IL-3 was purified to homogeneity from material obtained from WEHI-3 cells (2), and the gene encoding it was isolated (3, 4).

rIL-3 and IL-3 purified to homogeneity have been shown (5–7) to promote growth of precursors of various lineages of the hematopoietic system. While the presence of receptors for IL-3 on the cell membrane of target cells was shown by radioimmunobinding assay using labelled IL-3 (8), and by the ability of cytokine-sensitive cells to absorb IL-3 (9), the biochemical characteristics of such receptors and the mechanism of action of IL-3 are not defined. To further study the nature of the cells sensitive to IL-3, and to learn about the mechanism of action of this growth factor, we have attempted to develop mAb against IL-3-R. Here, we describe two mAb, termed CC11 and CB5, that appear to react against such receptors.

Materials and Methods

Animals. BALB/c, CBA/J, and BALB/c *nu/nu* mice (5–10-wk-old males and females) were purchased from the Institute for Biomedical Research, Füllinsdorf (Füllinsdorf, Switzerland).

Cells. The IL-3-dependent lines Ea3.15, Bc/Bm 11, CB/Bm7, Ba/Cl, L/B Ag A₂, 10T4⁻, L/BE₁E₅, Ba/Nu, Sj/B, SJ/L, Ba/B₂, NB/W, NZ/S were described elsewhere (9, 10). The IL-3-dependent lines FL/Cl, FL/A3, FL/d8 were established from fetal liver (BALB/c, 14th day of gestation), and their characteristics will be described elsewhere. The IL-3-sensitive 4c2 myeloid cells were provided by Dr. J. Garland (University of Manchester, Manchester, United Kingdom) and the IL-3-dependent 32DC123 myeloid line was provided by Dr. J. N. Ihle (Frederick Cancer Research Institute, Frederick, MD). The LBRM-33, clone 1A5, IL-1-sensitive T lymphoma was a gift of Dr. J. Watson (University of Auckland, Auckland, New Zealand). The P388D1, J771-D4, NIH-3T3, L929, Raw 8.1, and P815 lines were obtained from the American Type Culture Collection (Rockville, MD). EL-4, 70Z/3, WEHI-279, and A20.2J lines were provided by Dr. T. Leanderson (Basel Institute for Immunology). BW5147, CTLL, CFL1, and FLH1.25.5 lines were supplied by Dr. W. Haas (Basel Institute for Immunology). The 18.81 Abelson virus-transformed pre-B cell lymphoma was supplied by Dr. K. Marcu (New York University Medical Center, New York), and the Bc16 pre-B lymphoma was supplied by

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Dr. J. McKearn (Basel Institute for Immunology). T cells from BALB/c mice were obtained by passing spleen cells through nylon wool columns (11), and IgM-bearing spleen B lymphocytes were isolated by panning, as described (10). Cell suspensions from fetal liver and bone marrow were prepared as described before (10, 12). Cells from thymuses and lymph nodes (mesenteric) were obtained as recommended by Mishell and Shiigi (13). Spleen cells (2×10^6 cells/ml) were activated by Con A ($2 \mu\text{g/ml}$; Pharmacia Fine Chemicals, Uppsala, Sweden) or LPS ($50 \mu\text{g/ml}$; Difco Laboratories, Detroit, MI) at 37°C for 48 h in 50 ml tissue culture flasks (A/S Nunc, Roskilde, Denmark).

Cytokines. rIL-2 (14) was a gift of Dr. W. Fiers and Biogen (Geneva, Switzerland), mouse rIL-3 (3) was a gift of Dr. T. Yokota and DNAX (Palo Alto, CA), purified IL-1 and purified colony stimulating factor of granulocytes and macrophages (GM-CSF)¹ were purchased from Genzyme (Suffolk, England). B cell maturation factor (BMF), constitutively secreted by the LD1 T helper line (15) was prepared as follows: 2×10^6 LD1 cells in 1 ml of culture medium (Iscove's Dulbecco-modified medium [IDMM] plus 2% heat-inactivated human AB serum, gentamycin ($50 \mu\text{g/ml}$), and 2-ME (5×10^{-5} M) were incubated at 37°C for 3–4 d. The supernatants were collected, filtered ($0.2 \mu\text{m}$ Acrodisc filters), and assayed for BMF activity as detailed below. Aliquots of active supernatants were taken and stored at -20°C . They did not contain detectable IL-1, IL-2, IFN- γ , or B cell growth factor (BCGF) activities. Supernatant from spleen cells (BALB/c and CBA/J mice) stimulated with Con A (Con A-Sup) were prepared as described before (15).

Assays for Cytokine Activity. IL-1 was tested by its capacity of promoting the production of IL-2 by LBRM-33, clone 1A5, cells in the presence of PHA (Wellcome Research Laboratories), as described by Gillis and Mizel (16). IL-2 and IL-3 were tested for their capacity of supporting growth of IL-2-dependent CTL (CTLL) (10^4 cells/well) and IL-3-dependent Bc/Bm 11 (10^4 cells/well) cells as described in detail elsewhere (15). BMF was assayed as follows: spleen cells from BALB/c *nu/nu* mice (10^5 cells/well) suspended in microplate wells (A/S Nunc) in a final volume of $200 \mu\text{l}$ of culture medium containing optimal concentrations of LD1 supernatant (final concentration 10–15%) were incubated in duplicate at 37°C for 5 d. The number of IgM-secreting cells was determined by the protein A reverse hemolytic plaque assay (17). Cell proliferative responses were measured by [^3H]thymidine uptake ($1 \mu\text{Ci/well}$, sp act 5 mCi; Radiochemical Center, Amersham, United Kingdom) during the last 6 h of a 24-h culture period conducted at 37°C .

Mitogen-initiated Proliferation and Antibody Secretion of Spleen Cells. Spleen cells from CBA/J or BALB/c mice (10^5 cells/well) suspended in microplate wells in a final volume of $200 \mu\text{l}$ of culture medium were stimulated by Con A ($2 \mu\text{g/ml}$), PWM ($1 \mu\text{g/ml}$), or LPS ($50 \mu\text{g/ml}$) at 37°C . Cell proliferation was measured by [^3H]thymidine uptake during the last 15 h of a 4-d culture period, and the number of IgM-secreting cells was measured by the protein A plaque assay after 5 d of culture.

Development of CC11 and CB5 mAb. The IL-3-R⁺ Ea3 cells (10) were injected (2×10^7) i.p. into a BALB/c mouse, and this procedure repeated twice at intervals of 3 wk between each injection. 4 d after the last boost, splenocytes were isolated and fused with the HAT-sensitive non-Ig secretor myeloma PAI-O (a gift of Dr. H. Jacot, Hoffmann-La Roche, Basel, Switzerland) at a ratio of 5:1 using a 50% (wt/vol) solution of PEG (4,000 mol wt). The cells were distributed in 96 microplate wells in a final volume of $200 \mu\text{l}$ of HAT selective medium. 10–15 d later, wells containing growing hybridomas were screened for reactivity against IL-3-sensitive clones by an indirect radioimmunobinding assay using ^{125}I -labelled F(ab)₂ rabbit anti-total mouse Ig (New England Nuclear, Boston, MA) as second antibody. 5 out of 1,340 wells screened were found to specifically react against IL-3-sensitive cells. Two of these five hybridomas are described here; hybridomas CC11 and CB5 were cloned and recloned by limiting dilution, (0.5 cells/microplate well) and are maintained in culture medium in tissue culture flasks. The type of Ig secreted by CC11 and CB5 hybridomas was identified by double-diffusing agar gels using goat anti-mouse IgG1, IgG2, IgG3, and IgM antisera (Nordic, Tilburg, the Netherlands). CC11

¹ *Abbreviations used in this paper:* BMF, B cell maturation factor; Con A-Sup, supernatant of spleen cells stimulated with Con A; GM-CSF, colony-stimulating factor of granulocytes and macrophages; IDMM, Iscove's Dulbecco-modified medium.

and CB5 hybridomas secrete mouse IgG2, which bind to protein A, and fix rabbit complement. CC11 and CB5 mAb were purified from hybridoma culture supernatants by protein A-Sepharose column chromatography (Pharmacia Fine Chemicals). They were dialyzed, filtered, and stored at -70°C until used.

Other Antibodies. The following mAb were used in the form of hybridoma culture supernatants: M1/42 (18) antibody reactive with H-2 antigens of all haplotypes; 34-2-12 S (19) antibody against H-2-D^d. Both hybridomas were obtained from the American Type Culture Collection. 14.8 (20) antibody against the B-220 molecule was kindly provided by Dr. P. Kincade (Oklahoma Medical Center, OK).

Purification of CC11/CB5⁺ and CC11/CB5⁻ Bone Marrow Cells. Bone marrow cell suspensions were prepared from femurs and tibia of BALB/c mice as detailed elsewhere (12). The cells were washed two times in balanced salt solution (BSS) and resuspended in separation medium (IDMM with 5% FCS, gentamycin, and 2-ME) at 6×10^7 cells in 3 ml. The cells (3 ml of cell suspension) in 50 ml conical plastic tubes (2070 F; Falcon Labware, Oxnard, CA) were exposed to CC11 or CB5 mAb (70 $\mu\text{g}/\text{tube}$) at 4°C for 30 min. The tubes were centrifuged (5 min, 800 g), and the supernatants were discarded. The cells in the pellet were resuspended by gently shaking the tubes, and they were mixed with a suspension of protein A-coupled SRBC (final dilution 1:25) in a final volume of 5–6 ml of separation medium. The tubes were spun (5 min, 800 g), and incubated at 4°C for 45–60 min. The pellet was gently resuspended with wide-bore Pasteur pipettes and layered over a 3-ml Ficoll-Hypaque gradient contained in round-bottom plastic tubes (2057; Falcon Labware), and the tubes were centrifuged at 4°C (40 min, 1,100 g). Non-rosette forming cells were collected from the interphase, and rosette-forming cells in the pellet were resuspended gently in separation medium and centrifuged again over Ficoll-Hypaque gradients as indicated above. Rosette-forming cells in the pellet of the second gradient were collected, the erythrocytes were lysed with ammonium chloride-Tris buffer, and the remaining viable cells were washed in separation medium and resuspended in culture medium (see above) at desired concentrations. The non-rosette forming cells obtained in the first gradient were treated with CC11 or CB5 mAb (40 $\mu\text{g}/\text{ml}$, depending on which antibody was used in the separation) plus low-toxicity rabbit complement (final dilution, 1:12; Cedarlane Laboratories) to further deplete these cells of CC11/CB5⁺ cells. The cells were washed and resuspended in culture medium at desired concentrations. Both populations were then cultured at 37°C for 12–18 h to allow both shedding of the antibodies and reexpression of the CC11/CB5 glycoprotein.

Purity of the cell populations was determined by immunofluorescence microscopy (200–300 cells analyzed) using both CC11 and CB5 mAb as first antibody, and FITC-conjugated F(ab')₂ rabbit anti-mouse IgG antibody as second antibody. Non-rosette forming cells contained $\leq 1\text{--}4\%$ CC11/CB5⁺ cells, and rosette-forming cells had 96–98.7% CC11/CB5⁺ cells. The experiment described in Fig. 5 was conducted with a preparation of cells where we could obtain highly purified CC11⁺ ($\geq 98.7\%$ CC11/CB5⁺ cells) and CC11⁻ ($\leq 1\%$ CC11/CB5⁺ cells) cell populations. CC11/CB5⁺ and CC11/CB5⁻ cells (5×10^4 cells/well) were placed in round-bottom microplate wells in the presence or absence of the following stimuli: purified IL-1 (20 U/ml), rIL-2 (20 U/ml), rIL-3 (20 U/ml), or Con A-Sup (final concentration 25%) in a final volume of 200 μl of culture medium per well. The cultures in triplicate were incubated at 37°C for 48 h. Cell proliferation was determined by [³H]thymidine uptake (1 $\mu\text{Ci}/\text{well}$) during the last 8 h of the culture period.

Immunofluorescence Staining and Flow Fluorometry. The different types of cells ($3\text{--}5 \times 10^5$), harvested in logarithmic phase of growth, were incubated with purified CC11 and CB5 mAb (15 $\mu\text{g}/\text{tube}$), or with the M1/42 rat anti-H-2 antigen antibody (positive control) at 4°C for 30 min. The cells were washed once and resuspended in buffer (PBS plus 0.1% BSA plus 0.1% NaN₃) containing an appropriate dilution of FITC-conjugated F(ab')₂ rabbit anti-mouse IgG or rabbit anti-rat IgG at 4°C for 30 min. Negative controls were cells incubated in second labelled antibody only. The cells were washed twice in buffer, and a third time in buffer containing propidium iodine to exclude dead cells from analysis. Fluorescence intensity was measured by a FACS-II (Beckton Dickinson Immunocytometry

Systems, Mountain View, CA) equipped with an argon ion laser. Data were collected from 10^4 cells analyzed in 1024 channels using logarithmic amplification of fluorescence emitted by single viable cells.

Effect of CC11 and CB5 mAb on Various Cellular Functions. The effects of CC11 and CB5 mAb on the action of IL-1 (20 U/ml), IL-2 (20 U/ml), IL-3 (1–100 U/ml), BMF (final concentrations 10–40%); proliferative responses to Con A, PWM, and LPS; and on maturation of spleen B cells into antibody-secreting cells driven by LPS were studied by adding different concentrations of CC11 and CB5 mAb (0.1–40 μ g/ml) or control mAb M1/42, 14.8 (final concentrations 10–40%) at the beginning of the assays, carried out in triplicate as detailed above.

The capacity of IL-3 of modulating the expression of the CC11/CB5 glycoprotein on the cell membrane was studied as follows: Bc/Bm 11 IL-3-sensitive cloned cells in logarithmic phase of growth were incubated (2.5×10^5 cells in 12×75 -mm round-bottom plastic tube [2054; Falcon Labware]) in the presence or absence of 150 U of IL-3, IL-2, or GM-CSF at 37°C for 6–8 h. The tubes were centrifuged (5 min, 800 g), the supernatants were discarded, and the cells were tested for the presence of CC11, CB5, and class I MHC antigens (control) by immunofluorescence staining and flow cytometry as detailed above.

Cell Labelling and Immunoprecipitation. Cells (2×10^7 , viability >90%) were extensively washed with PBS, 1 mM CaCl₂, and suspended in 0.4 ml of the same buffer with 0.1 ml 200 mM sodium phosphate, pH 7.2, in a tube coated with 100 μ g Iodogen (Pierce Chemical Co., Rockford, IL). After addition of 1 mCi carrier-free Na¹²⁵I (100 mCi/ml; Amersham Corp.) the tube was shaken gently for 15 min on ice. The cells were pelleted at 3,000 g for 10 s, and washed three times with 1 ml cold PBS, 1 mM CaCl₂. The pellet was lysed in 1 ml lysis buffer, pH 7.2, containing 1% NP-40 (Fluka Biochemicals, Switzerland), 0.1% sodium deoxycholate, 20 mM sodium phosphate, 0.14 M sodium chloride, 1 mM PMSF, 0.3 TIU/ml aprotinin (Sigma Chemical Co., St. Louis, MO) and 0.05% Ovalbumin. After standing for 3 min at 0°C, the lysate was spun 3 min at 12,000 g, and the supernatant was further cleared at 50,000 g for 1 h.

Antibody was coupled to CNBr-activated Sepharose 4 B (Pharmacia Fine Chemicals) according to the manufacturers recommendations. In a typical experiment, 25 μ l antibody-Sepharose beads were incubated with 0.5 ml lysate in a vial, rolling over end for 1 h at 4°C. The beads were washed, with change of tubes, once with 1 ml and four times with 5 ml lysis buffer without the protease inhibitors. The final wash was with 3 ml of 20 mM Tris-HCl, pH 8.0.

Electrophoresis and Enzyme Treatment. Antigen was eluted from the Sepharose beads with 1% SDS sample buffer containing either 1% 2-ME or 4 mg/ml iodoacetamide, and boiled 3 min before SDS-PAGE. Two-dimensional electrophoresis was performed according to the procedure of O'Farrell (21), after eluting the antigen with 9 M urea, 1% 2-ME, 2% ampholines, pI 9–11 (LKB, Bromma, Sweden), and 2% NP-40. For treatment with endoglycosidase F, the beads were boiled 3 min in 50 μ l sample buffer containing 0.5% SDS, 1% 2-ME, 10 mM sodium EDTA, 10 mM Tris-HCl, pH 8.8. 5 μ l 10% NP-40 and 5 μ l endoglycosidase F (kindly supplied by Dr. J. Kaufman, Basel Institute for Immunology) with 1 mM PMSF and 0.3 TIU/ml aprotinin were added and incubated 3 h at 37°C. Under these conditions, the proteolysis observed with ¹²⁵I-BSA as substrate was negligible. 15 μ l of a solution containing 10% SDS, 40% 2-ME, and 50% glycerol were added, and the samples were boiled again before SDS-PAGE. [¹⁴C]Methylated proteins (Amersham Corp.) were used as molecular weight markers.

Results

The hybridomas CC11 and CB5 were obtained by fusing splenocytes from a BALB/c mouse (repeatedly immunized with IL-3R⁺ Ea3 cells) with the HAT-sensitive myeloma PAI-O. Both CC11 and CB5 hybridomas secrete mouse IgG2b κ , which bind to protein A and which fix rabbit complement. All the experiments described below were carried out with purified CC11 and CB5 mAb.

Specificity. The specificity of CC11 and CB5 mAb was defined by testing their reactivity against a large panel of normal cells, growth factor-dependent lines and tumor cells representing various lineages by both immunofluorescence staining and flow fluorometry and indirect radioimmunobinding assays. All 18 IL-3-dependent mouse cell lines derived from spleen, bone marrow, and fetal liver tested, the pre-B cell lymphomas (70Z/3, 18.81, Raw 8.1) studied and the mastocytoma P-815 carried both CC11 and CB5 antigens on their cell membrane. The following type of cells were negative for CC11 and CB5 antigens: thymomas (EL-4, BW5147), IL-2-dependent T cell lines (CTLL, CFL1), the cytolytic T cell hybrid FLH 1.25.5, IL-1-sensitive LBRM-33 (clone 1A5) T lymphoma, macrophage tumor cells (P388D1, J771-D4, WEHI-3), surface Ig⁺ B lymphomas (BCL1, WEHI-279, A20.2J), fibroblast cell lines (NIH3T3, L929), purified spleen T cells, Con A-activated splenocytes, an enriched population of normal IgM⁺ B lymphocytes from spleen, and LPS-activated splenocytes. In all cases, cells that had the CC11 antigen were also positive for the CB5 antigen, and the reverse, CB5⁻ cells did not express CC11 antigen. Fig. 1 shows some examples to illustrate these results.

Tissue Distribution. We studied the presence of CC11⁺ and CB5⁺ cells in various tissues from normal mice by immunofluorescence staining and either microscopy or flow cytometry analysis. ~47–53% ($n = 3$) nucleated cells from fetal liver (14th day of gestation) of BALB/c mice, 61–79% ($n = 7$) nucleated bone marrow cells from 6–8-wk-old (CBA/J, BALB/c) mice, but only 3–10% ($n = 3$) splenocytes from young adult BALB/c mice were positive for both CC11 and CB5 antigens. Thymus, lymph node, heart, and kidney from young adult mice were negative ($\leq 1\%$).

Biochemical Characterization. The CC11 mAb precipitates from surface ¹²⁵I-labelled IL-3-dependent clones a protein that runs as a very diffuse band with an M_r of 50,000–70,000 under reducing conditions as determined by SDS-PAGE (Fig. 2a, lane A). Depending upon exposure time, the band resolved into two diffuse species (Fig. 2b, lane A), which were also present in the alkylated sample under nonreducing conditions (not shown). CC11 mAb did not precipitate similar species from radioactively labelled control EL-4 cells (Fig. 2a, lane F and b, lane E). The 75,000 M_r band present in precipitates from EL-4 cells with CC11 mAb was not observed in all the experiments. In the instances where it was present (such as in the experiment depicted in Fig. 2) the band showed a very different mobility before and after deglycosylation, and had a different pI than the antigen precipitated from both IL-3-dependent clones and freshly isolated bone marrow cells (Fig. 2, a and b, and other data not shown). Furthermore, in immunoprecipitations from the same labelled EL-4 cells with mAb of different specificity than that of CC11/CB5 mAb, a similar 75,000 M_r band was observed again in some but not all the experiments. Thus, it was not specifically precipitated by CC11 and CB5. Rather, it coprecipitated nonspecifically from labelled EL-4 cells under the conditions studied. Accordingly, the CC11/CB5 glycoprotein was not detected (four separate experiments) on the cell membrane of the same EL-4 cells, as determined by immunofluorescence and flow fluorometry analysis (Fig. 1, i).

The other control for the specificity of the molecule precipitated by CC11 was

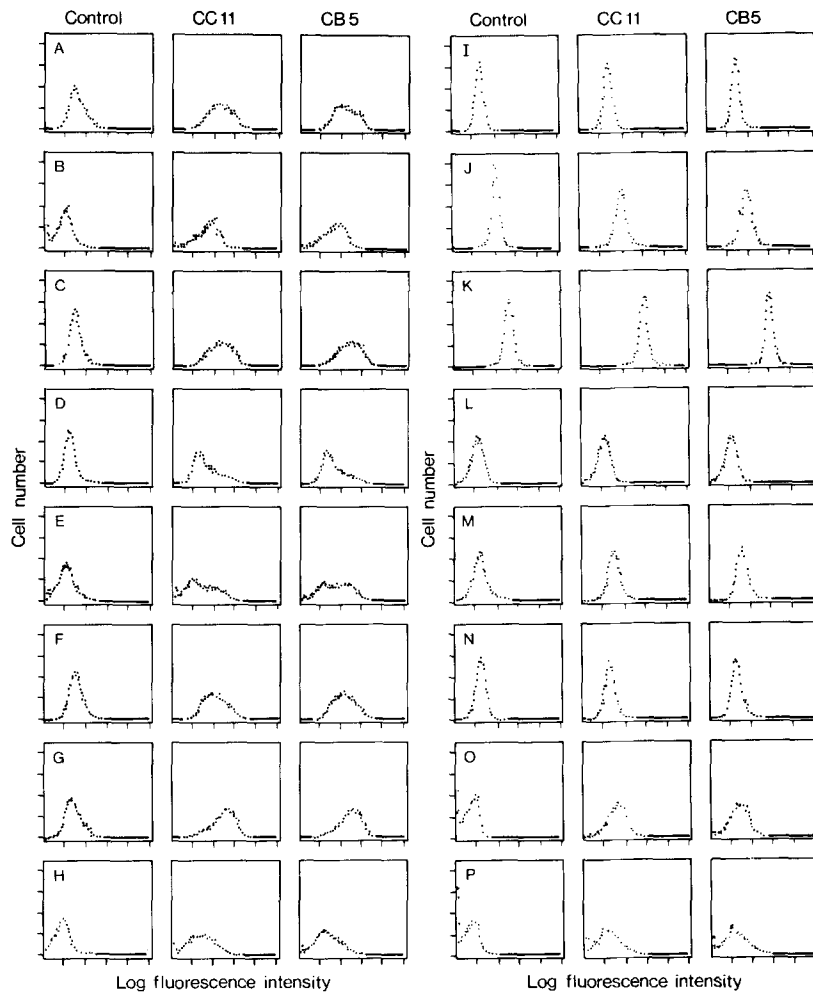


FIGURE 1. Specificity of mAb CC11 and CB5 for mouse IL-3-sensitive cells. The specificity of the CC11 and CB5 was determined by studying their reactivity against a large panel of cells representing several lineages and obtained from different tissues by immunofluorescence staining and flow fluorometry. Some fluorescence histograms generated in these studies are only shown here to illustrate the results obtained. They are: (A) Ea3.15 IL-3-sensitive clone (BALB/c); (B) CB/Bm7 IL-3-sensitive B stem cell (CBA/J); (C) Bc/Bm 11 IL-3-sensitive B stem cell (BALB/c); (D) 4C2 IL-3-sensitive myeloid line (BALB/c); (E) freshly isolated nucleated cells from fetal liver (BALB/c 14th day of gestation); (F) 10T4⁻ IL-3-sensitive pre-B cells (B10T.GR); (G) 32 dcl IL-3-sensitive myeloid cells (C₃H/HeJ); (H) L/B AgA2 IL-3-sensitive B stem cell (MRL/lpr); (I) EL-4 thymoma; (J) P388D1 macrophage; (K) WEHI-279 B lymphoma; (L) L929 fibroblast cells; (M) LBRM-33, clone 1A5, IL-1-sensitive T lymphoma; (N) CTLL IL-2-sensitive T cells; (O) Raw 8.1 pre-B lymphoma; (P) 18.81 pre-B lymphoma.

that M1/42 anti-H-2 precipitated from IL-3-dependent Bc/Bm 11 cells proteins with M_r compatible with that of H-2 antigens, but incompatible with that detected by CC11 or CB5 (data not shown).

The CC11 molecules from different cell populations show slightly different mobilities in SDS-PAGE, depending on the origin of the cells (Fig. 2a). Thus, the CC11 molecule of freshly isolated bone marrow cells often showed a slightly

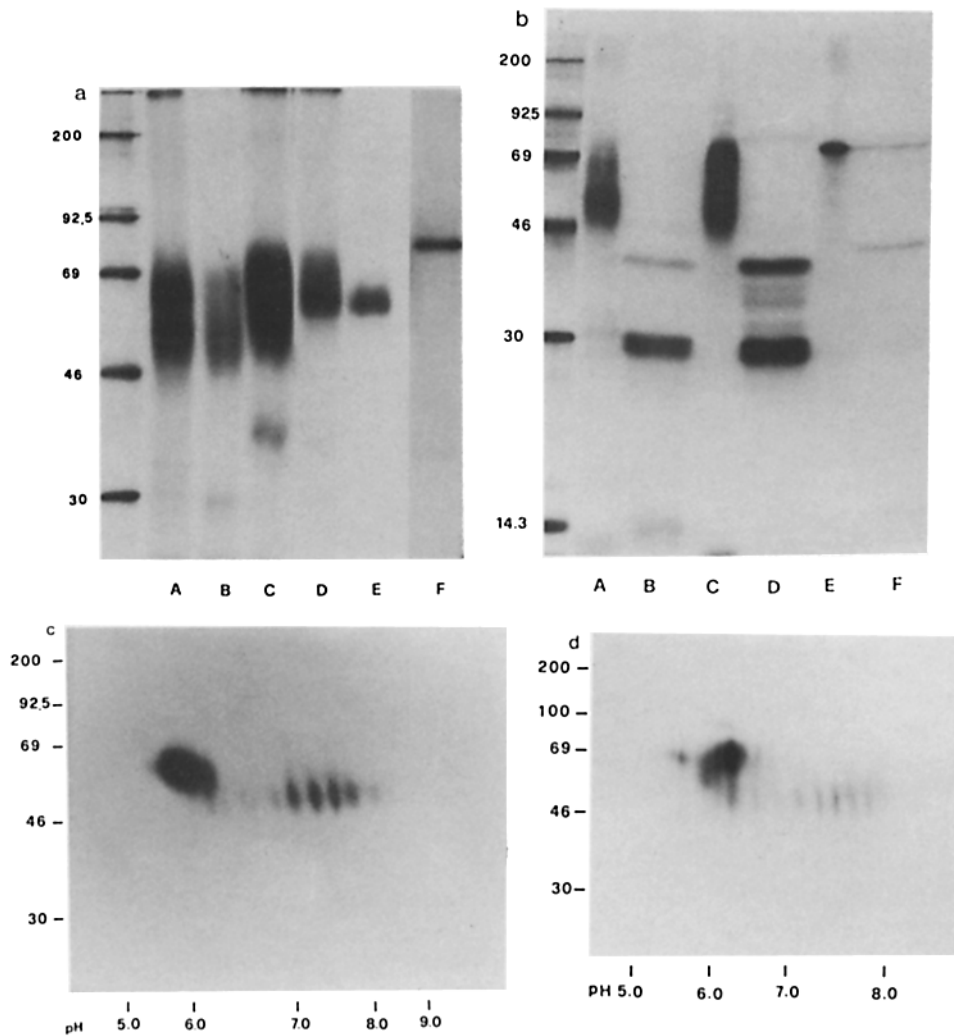


FIGURE 2. (a) SDS-PAGE analysis of immunoprecipitated material with mAb CC11 from surface ¹²⁵I-labelled cells. The lysate was adjusted to the same specific activity before precipitation. Lanes: A, Bc/Bm 11 cells; B and C, two different preparations of bone marrow cells; D, Raw8.1 cells; E, Bc16 cells; F, EL-4 cells. (b) SDS-PAGE analysis of immunoprecipitated material with mAb CC11 before and after treatment with endoglycosidase F. Lanes: A and B, Bc/Bm 11 cells; C and D, bone marrow cells (BALB/c); E and F, control EL-4 cells. (c) and (d) Two-dimensional electrophoresis (IEF followed by SDS-PAGE) of CC11 antigen precipitated from Bc/Bm 11 cells (c) and bone marrow cells (BALB/c) (d). Numbers to the left in each section are M_r ($\times 10^{-3}$) markers.

lower mobility than the CC11 protein of IL-3-dependent clones (Fig. 2a, lane C). Likewise, the CC11 molecule of Abelson virus-transformed pre-B lymphomas (Raw8.1, Bc-16) has similar mobilities to that of IL-3-dependent clones and bone marrow cells, but the lower M_r species of the molecule are not present in these cells (Fig. 2a, lanes D and E). Such heterogeneity in size of the CC11 molecule seems to be due mainly to differences in glycosylation. Indeed, treatment of the CC11 antigen of either IL-3-dependent Bc/Bm 11 cells or bone marrow cells

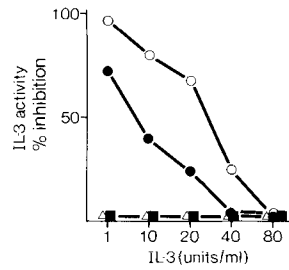


FIGURE 3. CC11 and CB5 inhibit IL-3-driven growth of cytokine-dependent clones. Bc/Bm 11 cloned cells were incubated in microplate wells in the presence of CC11 (○), CB5 (●), (2.0 $\mu\text{g}/\text{ml}$), control antibodies M1/42 (△), 14.8 (■) (final concentration 30% vol/vol) or medium only at 22°C for 10 min. Different concentrations of rIL-3 were added to the cultures in a final volume of 200 μl /well. Cell proliferation was measured by [^3H]thymidine uptake during the last 6 h of the culture period. The results are expressed as percent inhibition of IL-3-driven proliferation, and were calculated as: percent inhibition = $1 - (\text{cpm from cultures established with mAb}) \times 100 / (\text{cpm from cultures established without mAb})$.

with endoglycosidase F yields a band of $M_r \sim 40,000$, and a doublet at 30,000 and 28,000 on both types of cells, probably representing partially and totally deglycosylated material, respectively (Fig. 2*b*, lanes *B* and *D*). Two-dimensional electrophoresis shows that the CC11 antigen of either IL-3-dependent Bc/Bm 11 cells (Fig. 2*c*) or bone marrow cells (Fig. 2*d*) focuses between pH 5.7 and 6.2, which is accompanied by four weak bands at a lower molecular weight of pH 7.0–8.0, probably reflecting different degrees of sialylation.

The molecules recognized by CC11 and CB5 are identical by SDS-PAGE analysis on all cells tested.

Functional Relationship to the Action of IL-3. The possible functional relationship between the CC11/CB5 glycoprotein and the action of IL-3 was investigated in three different ways. First, we tested the effect of mAb CC11 and CB5 on the growth of cloned cell lines supported by IL-3. CC11 inhibited the proliferative response promoted by IL-3. 2 $\mu\text{g}/\text{ml}$ of the antibody inhibited by ~60–70% the proliferation of Bc/Bm 11 cells supported by 10 U/ml of rIL-3 (Fig. 3). This inhibitory effect was greater if lower concentrations were used, and could be overcome by higher concentrations of the growth factor (Fig. 3). mAb CB5 exhibited less inhibitory activity than CC11 (Fig. 3 and Table I). The antibodies interfered with the action of IL-3 on different IL-3-sensitive cell lines obtained from various tissues (fetal liver, bone marrow, spleen) and derived from different strains of mice. The inhibition of IL-3-driven growth caused by mAb CC11 and CB5 is specific. mAb against other antigens present on the cell membrane of the same target cells (eg. H-2, B-220) did not affect their growth supported by IL-3 (Fig. 3). Moreover, neither CC11 nor CB5 showed inhibitory or stimulatory effects on the action of IL-2, purified IL-1, or B cell maturation factor (BMF); nor on the proliferative responses of splenocytes initiated by Con A, PWM, and LPS; nor on the maturation of spleen B cells into antibody-secreting cells stimulated by LPS (Table I). Finally, CC11 and CB5 do not by themselves support growth of IL-3-dependent cells, either in soluble form or linked to Sepharose beads (R. Palacios, unpublished results).

Second, we tested whether IL-3 would modulate the glycoprotein recognized

TABLE I
CC11 and CB5 mAb Inhibit Action of IL-3 But Not of Other Cytokines

| mAb | Biological Activity | | | | | | | |
|-----------------------------|-------------------------------|--------|--------|-------------------------------|---------|---------|--------------|--------------------|
| | IL-1 | IL-2 | IL-3 | BMF (IgM PFC/culture) | Con A | PWM | LPS | |
| | | | | | | | Δ cpm | IgM PFC/culture |
| <i>μg/ml</i> | <i>Δcpm</i> | | | <i>Δcpm</i> | | | | |
| None | 19,721 | 23,737 | 20,107 | 8,400 | 199,250 | 136,817 | 74,509 | 13,500 |
| CC11 | | | | | | | | |
| 2.0 | 18,896 | 23,210 | 9,776 | 8,550 | 201,073 | 143,021 | 79,533 | 12,400 |
| 10.0 | 19,630 | 23,201 | 4,091 | 8,800 | 214,333 | 138,163 | 78,451 | 13,200 |
| 20.0 | 19,513 | 23,144 | 1,025 | 8,120 | 207,824 | 144,712 | 81,198 | 12,100 |
| CB5 | | | | | | | | |
| 2.0 | 19,710 | 23,112 | 15,695 | 8,080 | 217,772 | 130,578 | 73,128 | 15,040 |
| 10.0 | 18,818 | 23,635 | 10,187 | 7,950 | 206,263 | 141,126 | 76,066 | 12,500 |
| 20.0 | 19,601 | 23,025 | 4,351 | 8,600 | 218,503 | 138,025 | 71,186 | 13,600 |

CC11 and CB5 mAb were tested for their activity on the action of purified IL-1 (20 U/ml), rIL-2 (20 U/ml), rIL-3 (10 U/ml), BMF (final concentration, 10%); proliferative responses of spleen cells to Con A (2 μ g/ml), PWM (1 μ g/ml), LPS (50 μ g/ml); and in the maturation of splenic B cells into antibody-secreting cells stimulated by LPS. Data are expressed as Δ cpm (cpm in stimulated cultures - cpm unstimulated cultures) for the proliferative responses, and as IgM PFC per culture for the antibody-production responses.

by CC11 and CB5. The IL-3-dependent Bc/Bm 11 cells were preincubated in rIL-3 at 37°C for 6–8 h. The same cells were also preincubated in rIL-2 or purified GM-CSF and served as controls. The cells were then assessed for the presence of CB5, CC11, and class I MHC (control) antigens by immunofluorescence staining and flow cytometry. The results (Fig. 4) showed that Bc/Bm 11 cells preincubated in IL-3 bound significantly less CC11 and CB5 than the cells preincubated in medium only (Fig. 4, *a* and *b*). IL-3 did not, however, affect the expression of class I MHC antigens on the cells (Fig. 4*b*). Nor did GM-CSF (Fig. 4*c*) or IL-2 (Fig. 4*d*) modulate the expression of the CC11/CB5 glycoprotein on the cells. Thus, IL-3 specifically modulates the expression of the CC11/CB5 glycoprotein on the cell membrane.

Third, freshly isolated bone marrow cells were separated into cells having (CC11⁺) and cells lacking (CC11⁻) the CC11 glycoprotein on their cell membrane, as detailed in Materials and Methods. After an incubation period at 37°C to allow both shedding of the antibody and reexpression of the CC11/CB5 glycoprotein, CC11⁺ and CC11⁻ cell populations were tested for their proliferative response to IL-3 and to other cytokines as well. The results, obtained in an experiment where we could obtain highly purified CC11⁺ ($\geq 98.7\%$ CC11/CB5⁺ cells) and CC11⁻ ($\leq 1\%$ CC11/CB5⁺ cells) cell populations are depicted in Fig. 5. While CC11⁺ cells proliferated in response to IL-3 CC11⁻ cells did not. Neither cell population showed significant responses to purified IL-1 or rIL-2, but both CC11⁺ and CC11⁻ cells proliferated in response to a crude supernatant containing various activities released by mouse spleen cells stimulated by Con A (ConA-Sup). Similar results were obtained when bone marrow cells were separated into

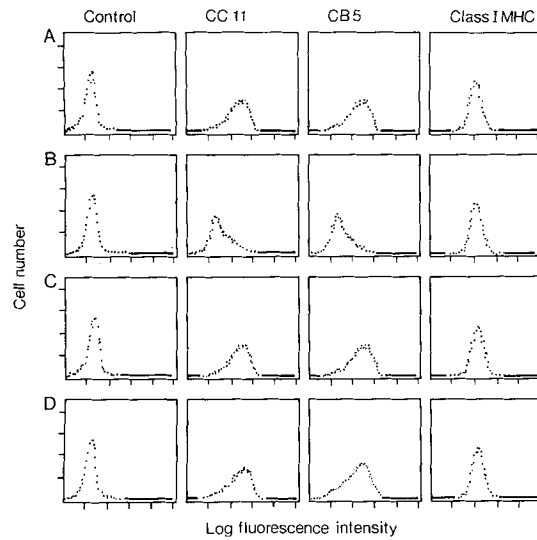


FIGURE 4. α IL-3 modulates the expression of the CC11/CB5 glycoprotein in the cell membrane. Bc/Bm 11 cells were preincubated in medium only (A), IL-3 (B), GM/CSF (C), or IL-2 (D) at 37°C for 8 h. The cells were then tested for the presence of CC11, CB5, and class I MHC antigens by immunofluorescence staining and flow cytometry. Histograms from cells of the different groups incubated with second labelled antibody only (controls).

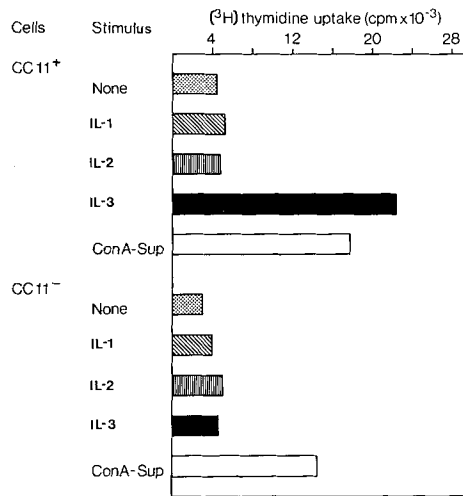


FIGURE 5. Bone marrow cells were separated into CC11⁺ and CC11⁻ cell populations and tested for their proliferative response to the agents indicated (final concentration of IL, 20 U/ml; Con A-Sup final concentration, 25%). Cell proliferation was determined by [³H]-thymidine uptake during the last 8 h of a 2-d culture period conducted at 37°C. The results are expressed as cpm × 10⁻³ (mean of triplicate samples). The SEM in this experiment was <8.3%.

CC11⁺ and CC11⁻ populations and with bone marrow cells from mice carrying the *xid* and nude mutations, which preclude development of mature T and B lymphocytes (22, 23) (data not shown). Thus, bone marrow cells that have the CC11/CB5 molecule on their cell membrane are sensitive to IL-3, whereas bone

marrow cells that do not express this molecule on the cell membrane are insensitive to IL-3.

Discussion

Evidence was presented in Results that strongly supports the view that mAb CC11 and CB5 may react against receptors for mouse IL-3. CC11 and CB5 (*a*) specifically inhibited the action of IL-3 on growth factor-dependent clones. (*b*) IL-3 specifically modulated the glycoprotein detected by mAb CC11 and CB5 on the cell membrane. (*c*) The CC11/CB5 glycoprotein is present on all IL-3-sensitive cell lines tested, but not on cells insensitive to IL-3. (*d*) CC11⁺ and CB5⁺ cells are well represented in tissues known to contain IL-3-sensitive cells (eg. bone marrow, fetal liver), but not in tissues where the majority of cells are insensitive to IL-3 (eg. adult thymus, lymph node, heart, kidney). (*e*) Freshly isolated bone marrow cells that have the CC11/CB5 glycoprotein on the cell membrane are sensitive to IL-3, while bone marrow cells that lack this molecule are not.

The alternative explanation for the observations described here is that the CC11/CB5 glycoprotein might not be the IL-3R *per se*, but it might be related to it. Although we cannot directly exclude this possibility, it seems to us less likely because one needs to further imply that the CC11/CB5 glycoprotein is linked to IL-3-R on the cell membrane (to explain its specific modulation by IL-3), and that it plays specifically a crucial role in the action of IL-3. We expect that, by isolating the gene encoding the CC11/CB5 glycoprotein, and using subsequent gene transfer experiments, we will be able to directly distinguish between these possibilities. In any event, it is clear that the CC11 and CB5 mAb specifically react against IL-3-sensitive normal mouse cells, and that the glycoprotein detected by them plays an essential role in the action of IL-3.

The CC11/CB5 molecule has an M_r of 50,000–70,000 by SDS-PAGE, and a pI of 5.7–6.2. The same species were observed under reducing and nonreducing conditions, excluding the presence of disulfide links between them. There is heterogeneity in size of the CC11/CB5 glycoprotein expressed on IL-3-dependent clones, bone marrow cells, and pre-B cell lymphomas. This is due mainly to differences in glycosylation. Treatment of the CC11/CB5 molecule with endoglycosidase F yields bands of M_r 40,000, 30,000, and 28,000 from material obtained from both IL-3-dependent clones and bone marrow cells. The different species probably represent partially and totally deaminoglycosylated material, respectively, or alternatively, might indicate the presence of two proteins. The data are most compatible with the view that the CC11/CB5 molecule is made of a single chain protein of 28,000 M_r , carrying heterogeneous *N*-linked glycans of high relative molecular weight. Nevertheless, at present we cannot exclude the possibility that this molecule is made of two chain proteins of different size. Further biochemical studies will hopefully distinguish between these possibilities. The heterogeneity in size of the CC11/CB5 glycoprotein of different cell populations is not a unique feature of this molecule. It is known that the same polypeptide can be glycosylated differently in different cell types (24). For example, receptors for several ligands (eg. C3, Fc, IL-2) have been found (25–27) to exhibit heterogeneity due mainly to differences in glycosylation.

Interestingly, pre-B cell lymphomas (18.81, Raw 8.1, Bc16) express the CC11/CB5 glycoprotein on their cell membrane. These results, and the findings that purified CC11/CB5⁺ bone marrow cells contain B cell precursors (surface Ig-negative) that proliferate and give rise in vitro to antibody-secreting cells after LPS stimulation (R. Palacios, manuscript in preparation) are consistent with the proposition that IL-3 acts on a population of mouse B cell precursors (9, 10). Studies are in progress to determine whether bone marrow cells committed to the T cell lineage express the CC11/CB5 glycoprotein. Thymocytes from fetal and adult thymuses have neither detectable CC11/CB5 antigens, nor do they use IL-3 as growth factor (R. Palacios unpublished results).

Freshly isolated nucleated cells from fetal liver and bone marrow cells have CC11/CB5 antigen, and proliferate in response to IL-3, suggesting that these cells may be using IL-3 in vivo. Mature T cells are known to synthesize IL3 (28), but these cells are not present in fetal liver, nor are they easily detectable in young adult T cell-deficient *nu/nu* mice. Yet these mice have normal (in bone marrow) and increased (in spleen) numbers of CC11/CB5⁺ cells as compared to euthymic mice, and both tissues contain cells that grow in IL-3 (R. Palacios, unpublished results). These observations raise the question what cells normally make IL-3 in fetal liver and bone marrow.

Finally, it is worth stressing that the CC11/CB5 glycoprotein is expressed not only on normal cells but also on their tumor counterparts. There is accumulating evidence that some oncogenes code for molecules resembling growth factors, their receptors, or molecules involved in the transduction of signals delivered by growth factors (29, 30). Possibly the CC11/CB5 protein is encoded by a protooncogene involved in transformation of haematopoietic cells. Our initial survey excluded the products of the *src*, *ras*, *abl*, *erb-B*, *mos*, *sis*, *fes*, *Myc*, *Myb*, *fos*, and *fms* as candidates, based on biochemical criteria and cellular location.

Summary

The mAb CC11 and CB5 reacted against all 18 IL-3-dependent cell lines tested, but not against cells insensitive to IL-3. Up to 53% nucleated cells from fetal liver (14th day of gestation) and 79% bone marrow cells of young adult mice were positive for both CC11 and CB5 antigens, but cells from thymus, lymph node, heart, and kidney were negative. The molecule recognized by both antibodies has an M_r of 50,000–70,000, a pI of 5.7–6.2, and carries heterogeneous *N*-linked glycans of high M_r . Both CC11 and CB5 specifically inhibited the growth of clones supported by rIL-3. Neither antibody affected the action of IL-1, IL-2, or B cell maturation factor; the proliferative responses of splenocytes to Con A, PWM, and LPS; nor the maturation of spleen B cells into antibody-secreting cells stimulated by LPS. rIL-3 specifically modulated the expression of the CC11/CB5 glycoprotein on the cell membrane of IL-3-dependent clones. Finally, freshly isolated bone marrow cells that have the CC11/CB5 glycoprotein on the cell membrane proliferated in response to IL-3, whereas cells that lack this molecule did not. We suggest that CC11 and CB5 react against receptors for mouse IL-3.

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