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Selection of BoCD25 monoclonal antibodies by screening mouse L cells transfected with the bovine p55-interleukin-2 (IL-2) receptor gene

J. NAESSENS, M. SILEGHEM, N. MACHUGH, Y. H. PARK,* W. C. DAVIS* & P. TOYE *ILRAD, Nairobi, Kenya and *Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, U.S.A.*

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SUMMARY

The bovine interleukin-2 receptor-alpha (IL-2Rα) gene has been isolated[1] and a rabbit antiserum against a fusion protein of the gene has been produced.[2] However, the antiserum does not inhibit IL-2-dependent proliferation. Since a panel of monoclonal antibodies (mAb) to bovine activation antigens was available, we transfected the gene into mouse L fibroblasts, selected stable transfectants with the rabbit antiserum, and screened for antibodies that bound the transfected cells but not the untransfected cells. Three mAb were selected and all three precipitated a molecule of M_r 55 000 (under reducing conditions) from activated cells, as expected from homology with mouse and human IL-2Rα (CD25, Tac). One of the three mAb was a strong inhibitor of IL-2-dependent proliferation of bovine lymphocytes. Thus, the availability of transfected cells allowed us to establish quickly and unequivocally the antigenic specificity of a number of antibodies.

INTRODUCTION

Two surface molecules mediate the binding of IL-2 to human and mouse cells: a 50,000 MW glycoprotein [CD25, Tac, interleukin-2 receptor-alpha (IL-2Rα)] which binds IL-2 with a low affinity,[3,4] and a 75,000 MW antigen (IL-2Rβ) which binds the interleukin with a slightly higher (intermediate) affinity.[5] The functionally important high-affinity receptor, which mediates cell proliferation, is a heterodimeric complex of both surface molecules.[6,7]

Unstimulated T and B lymphocytes can express IL-2Rα or IL-2Rβ in low quantities, but only after stimulation are high quantities of IL-2Rα, and to a lesser extent IL-2Rβ, observed together on the cell surface.[8] The IL-2Rα chain is also expressed on activated, but not resting monocytes.[9] The α chain is not found on natural killer (NK)-like cells, large granular lymphocytes or certain B lymphomas, although these cells express IL-2Rβ and are IL-2 responsive.[5,10,11]

Antibodies to bovine IL-2Rα would be useful tools for measuring the activation state of particular cells and for studying IL-2-dependent cell proliferation. The bovine IL-2Rα gene has been isolated[1] and a rabbit antiserum has been raised to a fusion protein of the molecule.[2] This antiserum detected bovine IL-2 receptors on *Theileria parva*-infected cells[2] and on stimulated lymphocytes (M. Sileghem, unpublished observation). However, this antiserum does not block IL-2-dependent proliferation.

We have transfected mouse L cells with the cloned bovine IL-2Rα cDNA,[1] and used these transfectants to screen existing panels of mAb to detect those specific for bovine IL-2Rα. This approach proved successful, and positive antibodies were used for analysis of their functional characteristics and for biochemical analysis of the receptor.

Abbreviations: Con A, concanvalin A; FBS, foetal bovine serum; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; mAb, monoclonal antibody; M_r, relative molecular mass; PAGE, polyacrylamide gel electrophoresis; PBMC, peripheral blood mononuclear cells; PWM , pokeweed mitogen; SDS, sodium dodecylsulphate.

Correspondence: Dr J. Naessens, ILRAD, PO Box 30709, Nairobi, Kenya.

MATERIALS AND METHODS

Production of mAb

At ILRAD, hybridomas were produced as described previously[12] by fusion of X63.Ag8.653[13] myeloma cells with spleen cells from mice immunized against bovine T-cell lines, *Theileria parva*-infected lymphocyte lines, or large B cells from trypansome-infected cattle.

Monoclonal antibodies that reacted with activated cells, but not with resting, unstimulated cells were further analysed and the cell distribution of their epitopes and kinetics of appearance after stimulation with lectins were established. All mAb suspected of reacting with the IL-2R were analysed on the transfected mouse cells.

The rabbit antiserum to a fusion protein of the bovine IL-2R was a gift from Dr D. A. E. Dobbelaere (Dept. of Parasitology, University of Bern, Switzerland).[2]

IL-2Rα gene

The IL-2Rα gene was originally cloned from a bovine cDNA library with the aid of the analogous human and mouse cDNA probes.[1] The *Eco*RI fragment containing the cDNA copy of the gene was subcloned from pTZ18U into pUC19 to allow subsequent subcloning of a *Pst*1 fragment of the gene into the *Pst*1 site of pCDM8.[14] The pCDM8 had been prepared by complete digestion with *Xho*1, removal of the stuffer region and religation. The final insert of the IL-2Rα gene in pCDM8 contained the entire coding region and 56 nucleotides of untranslated region 5′ to the initiation codon. The resultant plasmid was called pCD-boIL-2Rα.

Cells and cell lines

Ltk⁻ cells were a gift from Dr S. Alberti (Instituto Mario Negri, Milano, Italy). COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine and gentamycin at 50 μg/ml (complete DMEM).

Peripheral blood mononuclear cells (PBMC) were purified from blood by Ficoll–Paque (Pharmacia LKB, Uppsala, Sweden) centrifugation and several washes of Alsever's solution as described previously.[12] Cells at 2×10^6/ml were stimulated by concanavalin A (Con A) (Type V, Sigma Chemical Co., St Louis, MO) at 2·5 μg/ml or pokeweed mitogen (PWM) at (Sigma) 0·5 μg/ml, in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with L-glutamine, penicillin, streptomycin and 10% FBS.[12] After 4 days, activated cells were maintained by passaging them twice weekly at 5×10^5 cells/ml in fresh medium with 2·5 μg Con A or 0·5 μg PWM and 20% IL-2-containing supernatants. These IL-2-rich supernatants were recovered from 18-hr Con A-stimulated cultures of PBM.

Transfection

COS cells were transfected by the DEAE-dextran method. Cells grown to about 80% confluence in 75 cm² tissue culture flasks, were suspended by incubation in 'Trypsin-EDTA' (0·05% trypsin, 0·02% EDTA; Flow Laboratories, Irvine, Ayrshire, U.K.). After washing in complete DMEM and then in RPMI-1640 medium containing 50 mM Tris-HCl, pH 7·5, the cells were suspended at 10^6/ml in 3 ml RPMI-Tris containing 250 μg/ml DEAE-dextran (Sigma), 80 μM chloroquine (Sigma) and 3 μg/ml purified pCD-boIL-2Rα for 3 hr. The cells were washed, plated in 75 cm² flasks and analysed 60 hr after transfection.

The IL-2Rα gene was stably transfected into Ltk⁻ cells by calcium phosphate-mediated co-transfection[15] of pCD-boIL-2Rα (16 μg) with 2 μg of a plasmid containing the chicken thymidine kinase gene.[16] After 14 days of selection in complete DMEM containing hypoxanthine, aminopterin and thymidine, positive transfectants were selected by FACS using the rabbit anti-bovine IL-2Rα antiserum. After three rounds of sorting, the cells were cloned by limiting dilution, and used for mAb analysis and immune precipitation. The transfected cell line was called IL.X24.

Fluorimetry

Cells were indirectly stained with fluorescein isothiocyanate (FITC)-labelled anti-mouse Ig (Sigma) as described previously[17] and analysed on a FACStar PLUS (Becton Dickinson, Sunnyvale, CA).

IL-2R-transfected cells were stained with the rabbit antiserum in the absence of azide and FITC-labelled anti-rabbit Ig (Sigma) was used as a second step. About 17% of the cells were positive, and were sorted on the FACStar PLUS.

Immune precipitation

Immune precipitation was performed as previously described.[12,17] Iodinated blast cells, stimulated 3 days previously with Con A, or transfected mouse cells were lysed with NP-40 and the lysates precleared with protein A-sepharose (Pharmacia). Monoclonal antibodies that bound protein A were coupled to protein A-sepharose (50 μl ascites; 100 μl packed sepharose) by rotating the mixture overnight at 4°. After three washes with phosphate-buffered saline (PBS), the mAb-sepharose was added to the precleared lysates. For mAb that did not bind protein A, sepharose anti-mouse Ig was prepared by coupling 100 μl biotinylated anti-mouse Ig (Amersham, Amersham, Bucks, U.K.) to 100 μl packed streptavidin-sepharose beads (Sigma). Monoclonal antibodies were bound to this adsorbent under the same conditions as to the protein A-sepharose and were added to the lysates. After precipitation, the sepharose beads were washed several times, incubated in a reducing or non-reducing sample buffer and boiled at 100° for 5 min. The supernatants were analysed by SDS–PAGE and autoradiography.

Immunohistochemistry

Cryostat sections of bovine lymph node were stained by the indirect immunoperoxidase technique as previously described.[18]

Analysis of IL-2-dependent growth

Bovine PBMC were cultured in RPMI-1640 containing 10% FBS, 50 U penicillin/streptomycin/ml, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol (culture medium) and were stimulated with 5 μg Con A/ml (Sigma) for 3 days at 37° in a humidified atmosphere containing 5% CO_2. At the end of the incubation, the Con A blasts were harvested, centrifuged at 200 ***g***, incubated at 37° in medium for 1 hr and centrifuged over Ficoll–Paque (Pharmacia) at 4000 ***g*** to remove dead cells. Freshly prepared Con A blasts were suspended in culture medium and seeded in 96-well flat-bottomed tissue culture plates at a concentration of 10^5 cells per well. The cells were cultured with varying concentrations of the monoclonal antibodies and varying concentrations of recombinant human IL-2 (Amersham) for 3 days at 37° in a humidified atmosphere containing 5% CO_2. To analyse growth induced by natural bovine IL-2, supernatants from Con A-activated bovine PBMC were used instead of recombinant IL-2. The supernatants were supplemented with 20 mg/ml α-methyl-mannoside (Sigma). For analysis of cellular proliferation, the cultures were pulsed with 0·5 μCi ^{125}Iodo-2-deoxyuridine (Amersham) per well and harvested on glass filter paper (Skatron, AS, Lier, Norway) using a semi-automated cell harvester (Flow Laboratories). Incorporation of radiolabelled precursor was measured in a gamma-counter (Beckman Instruments Inc., Fullerton, CA). The means of triplicate values were calculated.

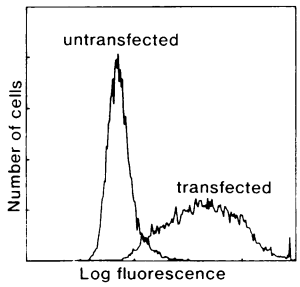

Figure 1. Fluorescence histograms of IL-2Rα-transfected and untransfected mouse L cells stained with CACT116A.

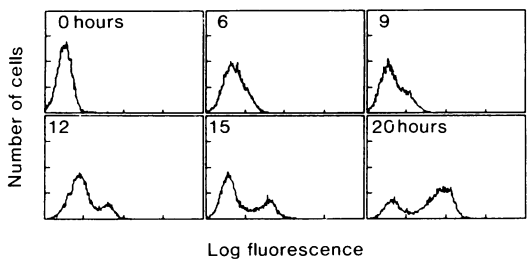

Figure 2. Kinetics of appearance of IL-2Rα expression on PBMC stimulated with 5 μg Con A/ml. Fluoresence histograms of PBMC stained with mAb IL-A111, at different times after lectin addition.

RESULTS

Stable transfection of the bovine IL-2Rα gene into Ltk⁻ cells

The bovine IL-2Rα gene was inserted into the eukaryotic expression plasmid, pCDM8, for transfection into COS and Ltk⁻ cells. To establish that the pCD-boIL-2Rα construct was functional, the plasmid was transiently transfected into COS cells. Indirect immunofluorescence using the polyclonal rabbit antiserum indicated that the IL-2Rα chain was expressed on the surface of transfected COS cells (results not shown).

To establish a cell line stably transfected with the bovine IL-2Rα gene, the pCD-boIL-2Rα plasmid was co-transfected into Ltk⁻ cells with a plasmid containing the chicken thymidine kinase gene. The HAT-resistant colonies were pooled and cells that stained positively with the rabbit anti-bovine IL-2Rα antiserum were sorted by FACS. Cloned cell lines were obtained by limiting dilution and monitored by FACS analysis. Figure 1 shows the FACS analysis of a cloned, transfected cell line (IL.X24) stained with the rabbit anti-IL-2R antiserum.

The IL.X24 cell line was then used to screen several mAb which were known to react with bovine activation antigens. This procedure established that three mAb, CACT108A, CACT116A and IL-A111 appeared to react with the bovine IL-2Rα.

Cellular distribution and kinetics

The mAb CACT108A, CACT116A and IL-A111 were used to study the expression of the IL-2Rα gene in normal lymphoid cells.

The antigen was not detected on resting peripheral blood cells, but it was expressed after Con A- or PWM-induced activation of these cells (Fig. 2). Expression of the antigen on the cell surface increased, as judged by increased fluorescence of labelled cells, from 10 hr to reach a maximum at Days 3 and 4, after which expression slowly decreased. The antigen was also detected on long-term cultured T-cell lines, on cloned $CD2^+$ $CD8^+$ cytotoxic T-cell lines, on $CD2^-$ $CD8^+$ non-major histocompatibility complex (MHC)-restricted cytotoxic T-cell lines (all cultured in the presence of IL-2) and most *T. parva*-transformed B and T cells, including γ, δ T-cell receptor$^+$ (TcR^+) cells (results not shown).

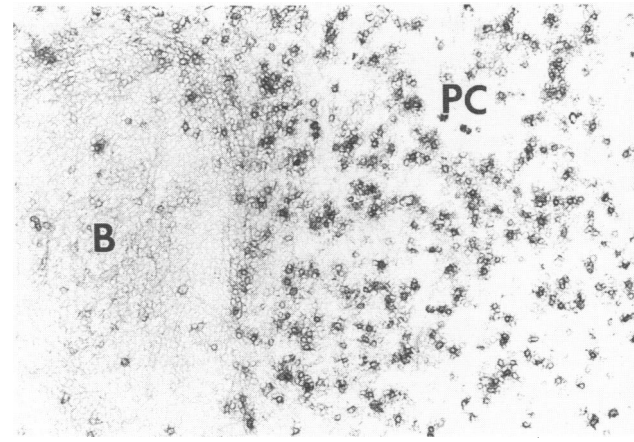

Figure 3. Immunoperoxidase staining of bovine lymph node with mAb IL-A111. Positive cells are concentrated in the T-cell-dependent paracortical region (PC), with a few positive cells in the B-cell follicle (B).

Labelling of cryostat sections of bovine lymph nodes with IL-A111 showed strong staining of some cells in the T-cell area (Fig. 3), but only a few cells in the follicles.

Biochemical analysis

Immune precipitation was performed on lysates from 3-day Con A-stimulated lymphocytes and from transfected cells. From the lymphocytes, mAb CACT108A, CACT116A and IL-A111 precipitated a single antigen, of M_r 55,000 when estimated under reducing conditions (Fig. 4). The antigen migrated faster under non-reducing conditions, with a M_r of 50,000.

An antigen of slightly smaller M_r (52,000 under reducing conditions) was obtained from the IL.X24 cells with CACT108A (Fig. 4). It is possible that post-translational modifications are different in the mouse cells, resulting in faster migration of the antigen isolated from transfected cells.

Inhibition of IL-2-dependent growth

To analyse the capacity of the mAb to inhibit IL-2-dependent growth, freshly prepared Con A-induced blast cells were incubated with or without antibody and stimulated with human recombinant IL-2 (rhIL-2). The proliferation was measured by incorporation of ^{125}Iodo-2-deoxyuridine 3 days after stimulation. The inhibitory capacity of the antibodies was analysed by comparison with an irrelevant mAb, BC9. Different concentrations of IL-2 and of antibody were used. The results in Fig. 5 show that IL-A111 blocks the IL-2-driven proliferation, and even at a high concentration of IL-2 (30 ng/ml) the antibody inhibited the proliferation by about 80%. At 3 ng (or about 1 U) of IL-2/ml, the antibody could be used at 1 μg/ml and still inhibit

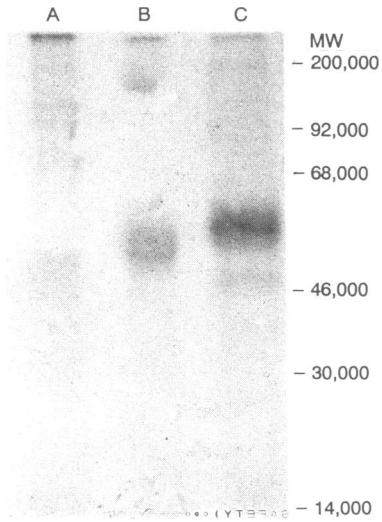

Figure 4. Immunoprecipitates from IL-2Rα-transfected cells analysed by 10% SDS-PAGE under reducing conditions with a negative control mAb (lane A) or anti-IL-2Rα mAb CACT108A (lane B), compared to immunoprecipitate from Con A-stimulated cells with the same mAb CACT108A (lane C).

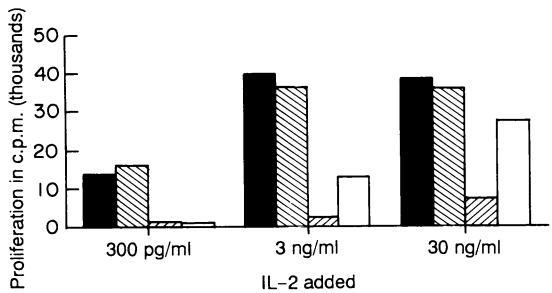

Figure 5. Inhibition of rhIL-2-dependent proliferation of bovine lymphocytes, measured by ^{125}Iodo-2-deoxyuridine uptake, by anti-IL-2Rα antibodies [IL-A111 (▨) and CACT116A (□)] and a control antibody [BC9 (▧)]; no mAb (■).

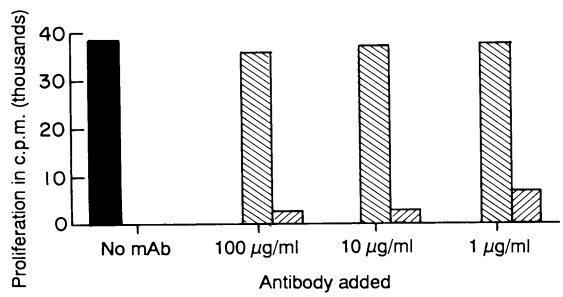

Figure 6. Capacity of different dilutions of mAb IL-A111 to inhibit rhIL-2-dependent proliferation of bovine lymphocytes. BC9 (▧); IL-A111 (▨); no mAb (■).

proliferation by about 80% (Fig. 6). In contrast, mAb CACT116A was a much less potent inhibitor, although it did block significantly when suboptimal levels of IL-2 were used (Fig. 5).

Since the affinity of the bovine IL-2R for rhIL-2 might differ from the affinity for natural bovine IL-2, we have tested the capacity of the mAb to inhibit cell proliferation with bovine IL-2-containing conditioned medium. Similar results were obtained: CACT108A and CACT116A were less effective inhibitors than IL-A111. The polyclonal rabbit antiserum raised against recombinant bovine IL-2R was tested in the same experiment, and was a very poor inhibitor: at a dilution of 1/40, a modest inhibition of 40% was noted. The preimmune rabbit serum also showed some inhibitory activity at higher concentrations (not shown).

Binding of radiolabelled rhIL-2 could not be inhibited by the mAb. This commercially radiolabelled available rhIL-2 (obtained from Amersham) contained an alanine-for-cysteine substitution at position 125. Similarly, proliferation of cells by unlabelled substituted rhIL-2 could not be inhibited by the mAb, even when high concentrations of IL-A111 were used. The substitution probably increased the affinity of IL-2 for the bovine receptor, making it impossible to inhibit without using very high concentrations of antibody.

DISCUSSION

A panel of mAb exists which recognizes antigens whose cellular distribution and kinetics of expression after Con A activation were similar to those of human IL-2Rα (CD25/Tac). We wished to establish that these mAb recognize the bovine equivalent of human IL-2Rα. However, the cellular distribution of an antigen is not sufficient evidence to prove homology between antigens of different species: in contrast to human and mice, MHC class II antigens in dogs, guinea-pigs and African buffalo are also expressed on resting T cells.[19,21] Porcine CD8 is also expressed on a large proportion of CD4$^+$ T cells.[22] In ruminants, CD44 has a much wider cell distribution than in mouse,[23] and there is evidence that IgD is not expressed at all on bovine B cells (J. Naessens and D. J. L. Williams, submitted for publication). Biochemical criteria, such as M_r or isoelectric point, can only suggest homology between antigens of different species, because many unknown surface molecules of the same size or charge might exist. Homology is only established after extensive functional tests or after showing sequence homology between the different products.

The bovine IL-2Rα gene had been cloned using mouse and human probes, and showed 71% homology with the human gene. We decided to establish the specificity of the candidate mAb by screening them against the expressed bovine gene product. Thus, Ltk$^-$ cells were transfected with a plasmid containing a cDNA copy of the bovine IL-2Rα gene and the stable, positive transfectants were used to test the panel of mAb. Three mAb were identified that bound to the transfectants, but not to the untransfected mouse cell line. The mAb recognized an antigen that appeared early after Con A activation (10 hr), showed a maximum expression on Days 3 and 4, and had a M_r of 55000 on SDS-PAGE under reducing conditions or 50000 under non-reducing conditions. Similar properties are displayed by the human and mouse IL-2Rα antigens.[3,4] In cryostat sections of bovine lymph node, mAb IL-A111 strongly stained some cells in the T-cell zones, as has been demonstrated for mAb to human IL-2Rα.[24]

The three mAb could inhibit proliferation of bovine lymphocytes induced by either rhIL-2 or supernatants containing bovine IL-2. However, CACT116A and CACT108 were significantly less potent than IL-A111 at inhibiting the proliferation of

stimulated cells. Indeed, at high IL-2 concentrations (30 ng/ml) CACT116A and CACT108A failed to inhibit significantly. Thus they either recognize an epitope that lies on the periphery of the IL-2-binding site or bind to the IL-2-binding site with a weaker affinity. The observation that blocking mAb could be raised by immunization with whole cells, but that very little or no inhibitory activity was found in the rabbit antiserum to the fusion protein, suggests that the IL-2-binding site was absent or not immunogenic in the fusion protein.

Producing transfectants allowed us to establish unequivocally the specificity of several mAb that were thought to react with bovine IL-2Rα (CD25). Similarly, if the genes encoding other bovine antigens were to be isolated, then it is possible to use transfection methodology to identify mAb specific for those antigens. Further, if no mAb exist, transfectants can be used as immunogens to produce mAb, as has been shown for human MHC class II antigens.[25]

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