Immunology 1986 **58** 351–358

Characterization by a monoclonal antibody and functional analysis of a subset of bovine T lymphocytes that express BoT8, a molecule analogous to human CD8

J. A. ELLIS, CYNTHIA L. BALDWIN, N. D. MACHUGH, A. BENSAID, A. J. TEALE, B. M. GODDEERIS & W. I. MORRISON *International Laboratory for Research on Animal Diseases, Nairobi, Kenya*

Accepted for publication 4 March 1986

SUMMARY

Monoclonal antibody (mAb) IL-A17 characterizes a subset of bovine T lymphocytes. IL-A17 recognizes a 34,000–35,000 MW doublet, designated BoT8, which is expressed on the surface of approximately 20% of peripheral blood mononuclear leucocytes (PBM), a subpopulation of lymphocytes in T-dependent areas of lymph nodes and spleen, and about 70% of thymocytes. This molecule is not expressed on B lymphocytes, monocytes/macrophages or granulocytes. Double labelling of PBM showed that the BoT8$^+$ population is distinct from the T lymphocyte subset expressing BoT4. BoT8$^+$ lymphocytes purified with a fluorescence-activated cell sorter (FACS) proliferated poorly in response to mitogenic and alloantigenic stimulation in the absence of exogenous growth factors. IL-A17 had no inhibitory effect on proliferation of PBM to mitogens (Con A and PHA) or alloantigens and no measureable effect on the *in vitro* generation of cytolytic effector cells. However, in some experiments IL-A17 was found to block partially allospecific cytolytic function mediated by bulk effector cell populations when included in ^{51}Cr-release assays. Fractionation of effector cells generated in an allogeneic mixed leucocyte culture (MLC) demonstrated that cytotoxic cells specific for class I major histocompatibility complex (MHC) antigens reside within the BoT8$^+$ population. Based on these data, and information reported elsewhere on alloreactive bovine T-cell clones, BoT8 is considered to be analogous to CD8 in humans and equivalent molecules in other species.

INTRODUCTION

Subpopulations of T lymphocytes have characteristic arrays of surface determinants, the expression of which is linked with their functional capacities (Lanier *et al.*, 1983). Monoclonal antibodies (mAb) recognizing antigens within differentiation clusters (IUIS-WHO Nomenclature Subcommittee, 1984) have demonstrated the evolutionary conservation of molecules defining functionally important T lymphocyte subsets among mammals. Two major subsets of T cells have been defined in human, mice, rats, pigs and sheep (Ledbetter *et al.*, 1981; Matsuura *et al.*, 1984; Pescovitz, Lunney & Sacchs, 1985; Maddox, Mackay & Brandon, 1985). In human and mice, the principal functional distinction between the two populations of lymphocytes is the restriction of their reaction to foreign antigen in combination with class I or class II major histocompatibility complex (MHC) molecules (Swain, 1983). In the case of humans, these subsets are defined by the expression of CD8 and CD4, respectively.

Detailed studies of the cellular basis of immune responses in cattle have been hampered by the paucity of markers for bovine leucocyte subpopulations. Recently, we have reported on the characterization of two mAb, IL-A11 and IL-A12, that recognize a molecule, BoT4, which has distribution, molecular mass and function similar to human CD4 (Baldwin *et al.*, 1986; Teale *et al.*, 1986). In the present communication, we describe the production and characterization of mAb IL-A17, which recognized a molecular, BoT8, on a subset of bovine T lymphocytes distinct from those expressing BoT4, and which have characteristics similar to lymphocytes expressing CD8 in humans and analogous molecules in other species.

Abbreviations: c.p.m., counts per minute; CTL, cytotoxic T lymphocytes; DPBS, Dulbecco's phosphate-buffered saline; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody(ies); MHC, major histocompatibility complex; MLC mixed leucocyte culture; PBM, peripheral blood mononuclear leucocytes; PMSF, phenyl methyl sulphonyl fluoride; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCGF, T-cell growth factor; TLCK, *N-p*-tosyl-L-lysine chloromethyl ketone; TRITC, tetramethyl rhodamine isothiocyanate.

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

MATERIALS AND METHODS

Production of monoclonal antibodies

Adult BALB/c mice were immunized intraperitoneally three times at 2-week intervals with 10^7 monocyte-enriched bovine peripheral blood mononuclear leucocytes (PBM) containing approximately 80% monocytes. Four days after the third inoculation, spleen cells were isolated and fused with X63-Ag8 myeloma cells essentially as described by Pearson *et al.* (1980). Supernatants from growing hybridomas were screened for reactivity with PBM by indirect immunofluorescence. Hybridomas from selected wells were cloned by limiting dilution in HAT medium using mouse thymocytes as feeder cells. Ascitic fluid was produced in BALB/c mice by conventional methods. The isotype of the mAb was determined by Ouchterlony immunodiffusion in 1% agar using supernatants from cloned hybridoma cultures and goat antisera specific for mouse immunoglobulin isotypes (Meloy, Springfield, VA).

Immunofluorescence staining and analysis with the fluorescence-activated cell sorter (FACS)

Indirect immunofluorescence staining was carried out as previously described (Lalor *et al.*, 1986). For two-colour immunofluorescence, PBM were reacted with IL-A17 (IgG1) and IL-A12 (IgG2a). Following incubation and washing, the cells were first incubated with biotin-conjugated goat anti-mouse IgG1 antiserum (Becton Dickinson, Sunnyvale, CA), rinsed, and then reacted simultaneously with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG2a antiserum and phycoerythrin-coupled avidin. The stained cells were analysed on a FACS (FACS II, Becton Dickinson). Cells were exposed to 488 nm light from an argon laser and the fluorescence emission was quantified at 525 nm for fluorescein and 575 nm for phycoerythrin at logarithmic amplification. The fluorescence signal was triggered on particles of size greater than platelets. The gate was set to exclude most dead cells and erythrocytes.

B cells were stained directly with goat anti-bovine IgM labelled with tetramethyl rhodamine isothiocyanate (TRITC) subsequent to indirect labelling with IL-A17 and FITC-conjugated anti-mouse Ig. Co-staining was then assessed by fluorescence microscopy.

Preparation of cell populations

Bovine PBM were obtained by Ficoll–Hypaque gradient centrifugation as described elsewhere (Lalor *et al.*, 1986). Populations of PBM depleted of, or enriched for, monocytes were obtained by defibrination plus depletion on plastic and adherence to plasma-coated gelatin, respectively, as previously described (Goddeeris *et al.*, 1986). Granulocyte-enriched populations were derived from the leucocyte-rich layer overlaying the pellet of erythrocytes formed during Ficoll–Hypaque gradient centrifugation. Contaminating erythrocytes were lysed with Tris-ammonium chloride buffer. Thymocytes were obtained by mincing thymic tissue in RPMI-1640 medium (Gibco Europe Ltd, Paisley, Renfrewshire) containing 10 μg/ml deoxyribonuclease (Sigma, Slough, Berks) and 10 IU/ml heparin, followed by passage through wire mesh sieves and two washes in medium.

In order to obtain populations of lymphocytes enriched or depleted of IL-A17$^+$ cells, to greater than 96% purity, for functional assays, monocyte-depeleted PBM were stained and sorted on the FACS as described previously (Baldwin *et al.*, 1986). Briefly, PBM were stained in bulk with IL-A17 (Lalor *et al.*, 1986) by indirect immunofluorescence, except that sodium azide was excluded from the staining medium and staining was conducted in 50-ml polypropylene centrifuge tubes. Unsorted control PBM were treated in the same manner but were not sorted with the FACS. In experiments requiring the addition of monocytes, PBM were similarly stained with IL-A24, a mAb that recognizes a cell surface determinant on monocytes/macrophages (J. A. Ellis, W. C. Davis, N. D. MacHugh and W. I. Morrison, in preparation) and sorted as above to obtain monocyte enriched populations.

Lymphocyte functional assays

PBM populations were stimulated *in vitro* with mitogens or allogeneic PBM as described elsewhere (Baldwin *et al.*, 1986). Briefly, PBM were cultured in flat-bottomed microtitre plates (Costar, Cambridge, MA) at 2×10^5 cells/well with 2 μg/well PHA.P (Difco, Detroit, MI) or 0·5 μg/well Con A (Sigma) in a final volume of 200 μl/well for 3 days at 39° in a humidified atmosphere of 5% CO_2 in air. For alloantigen responses, cells were cultured in the same manner except that 5×10^5 responder cells/well, $2{\cdot}5 \times 10^5$ gamma-irradiated (5000 rads) stimulator cells/well, and a 5-day culture period were used. FACS-sorted PBM were cultured in the same manner except that half-area microtitre plates (Costar) and half the cell concentrations and volumes of culture constitutents per well were used. In some experiments, bovine T-cell growth factor (TCGF) (Teale *et al.*, 1985) or purified monocytes were added at the initiation of the cultures to monocyte-depleted PBM so that the final concentration of monocytes was 10%. For blocking with mAb, ascitic fluid or purified immunoglobulin was added to the culture such that the final concentration was 50–100 times that required to give saturating levels of binding, as assessed by indirect immunofluorescence.

Alloreactive cytolytic T lymphocytes (CTL) were generated in bulk mixed leucocyte cultures (MLC) as previously described (Teale *et al.*, 1985). Animals had received allogeneic skin implants and had been boosted once after variable periods of time by a subcutaneous inoculation with $1{\cdot}5 \times 10^8$ PBM from the relevant skin donor. Responder PBM were obtained 3–12 weeks after the final immunization. Stimulator cells for MLC were irradiated PBM from the same animals used as donors for *in vivo* priming, or from animals matched for the class I MHC antigens of the skin donor. Cytotoxic activity of effector cells generated in MLC was assayed after 6 days of culture using a 4-hr ^{51}Cr-release assay with *Theileria parva*-infected lymphoblastoid target cells as described elsewhere (Teale *et al.*, 1985). In order to test for inhibition of generation of effector cells, mAb IL-A17 purified IgG was added at the time of initiation of the MLC. The capacity of antibodies to block cytolytic activity of effector cells was tested by incubation of effector cell populations in mAb at the same concentration as used for blocking of proliferative responses for 1 hr at 39° prior to adding the ^{51}Cr-labelled target cells.

In order to determine cytotoxic function of IL-A17$^+$ cells in bulk populations, effector cells obtained on Day 6 of MLC were first depleted of dead cells by centrifugation over Ficoll–Hypaque, then stained with mAb IL-A17 and sorted as described above. Cytotoxicity of these populations was measured using an ^{111}In-release assay conducted as described elsewhere (Shortman & Wilson, 1981). All effector populations

were tested on target cells that were matched or mismatched with stimulator cell populations with respect to class I MHC A locus products (Teale *et al.*, 1985). The target cell used did not express detectable levels of class II MHC antigens, as evaluated by staining with two mAb that react with bovine class II MHC molecules, namely R1 (Lalor *et al.*, 1986) and anti-HLA-DR (Teale *et al.*, 1986).

Immunohistochemistry

Cryostat sections (6–8 μm) were cut from snap-frozen blocks of various lymphoid organs and bone marrow from clinically normal cattle and collected on gelatin-coated slides (Van Ewijk, Van Soest & Van den Engh, 1981). Following air-drying for 1 hr and fixation in acetone for 10 min, sections were rehydrated in Dulbecco's phosphate-buffered saline (DPBS) and overlaid with 20% normal sheep serum in DPBS. The normal sheep serum was blotted off and the sections were reacted with mAb for 30 min. The sections were then rinsed twice for 10 min in DPBS, exposed to peroxidase-conjugated sheep anti-mouse Ig (Serotec Ltd, Bicester, Oxon) for 30 min and rinsed twice for 10 min in DBPS. The peroxidase conjugate was detected by reaction with 0·6 mg/ml diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO), 0·01% H_2O_2 and 0·01 M imidazole (Sigma) in Tris-buffered saline, pH 7·6 (Straus, 1982). All dilutions of antibodies were made in RPMI-1640 medium (Gibco) containing 2% gamma globulin-free horse serum and 30 mM NaN_3.

Immunoblotting

Briefly, 3–4 × 10^6 cells of a cloned population (T4·3) of IL-A17$^+$ bovine cytotoxic lymphocytes (CTL) (Teale *et al.*, 1986) were lysed in cold H_2O containing 1 mM phenyl methyl sulphonyl fluoride (PMSF) and 0·005 nM *N-p*-tosyl-L-lysine chloromethyl ketone (TLCK). Following sonication, 100 μl of buffer containing 0·186 M Tris-HCl, pH 8·0, 6% (v/v) sodium dodecyl sulphate (SDS), 15% (v/v) 2-mercaptoethanol (2-ME), 22·5% glycerol, and 0·03% (w/v) bromophenol blue (3 × sample buffer) were added. After boiling for 5 min, aliquots containing the equivalent of 10^5 cells were applied to discontinuous 7·5–17·5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) slab gels (Maizel, 1971). Following electrophoresis, proteins were transferred to nitrocellulose filters (BA 85, Shleicher and Schuell, Dassel, FRG) according to the method of Burnette (1981). The filters were then reacted with hybridoma culture supernatant according to the method of Shapiro, Voigt & Fujisaki (1986), dried and autoradiographed.

Immunoprecipitation

Cloned IL-A17$^+$ CTL (T4·3) or thymocytes were labelled with ^{125}I according to the lactoperoxidase/H_2O_2 technique of Ledbetter *et al.* (1981). Aliquots of 5 × 10^6 cells were incubated with IL-A17 ascitic fluid or an irrelevant IgG1 mAb diluted 1/100 in 1 ml RPMI-1640 (Gibco), washed once in RPMI-1640 and lysed in 1 ml of lysis buffer (Ledbetter *et al.*, 1981). Following centrifugation (15 min at 11,000 ***g***), antigen–antibody complexes were collected by the addition of 100 μl of 50% pre-swollen protein A-sepharose (Pharmacia Fine Chemical, Uppsala, Sweden), which had been precoated with affinity-purified rabbit anti-mouse Ig. The beads were washed five times in lysis buffer and immunoprecipitated molecules were released by boiling for 5 min in 100 μl three-fold concentrated sample buffer, with 2-ME (reduced) or without 2-ME (unreduced), and applied to 7·5–17·5% discontinuous SDS–PAGE slab gels (Maizel, 1971) prior to autoradiographic analysis.

RESULTS

Reactivity of IL-A17 with various cell populations

Although none of the hybrid supernatants reacted specifically with monocytes, the predominant cell type present in the immunizing population, several supernatants reacted with discrete populations of small to medium sized PBM. One hybrid, IL-A17 (IgG1), was analysed further. IL-A17 stained between 10% and 26% (mean 18%) of PBM in 28 cattle tested (Fig. 1a). These comprised 12 *Bos taurus* and 16 *Bos indicus*. In populations of cells highly enriched for monocytes, IL-A17 stained 3–4% of small to medium sized cells, which corresponded to the degree of lymphocyte contamination in these populations, as assessed by morphology and stained for alpha-naphthyl acetate esterase. In monocyte-depleted populations, IL-A17 stained a slightly higher number of lymphocytes than in routinely prepared PBM from the same animal. When PBM were co-stained with IL-A17 and TRITC-labelled goat anti-bovine IgM, IL-A17 reactivity was not found in the surface

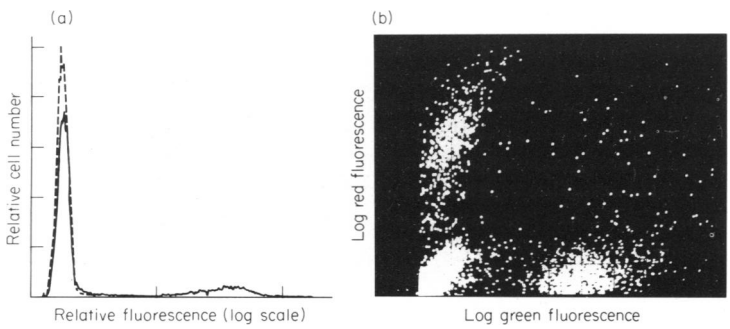

Figure 1. (a) FACS profile of PBM stained by indirect immunofluorescence with mAb IL-A17 (20·1% positive cells). Profile of PBM exposed to second-step antibody alone is indicated by broken line. (b) Dot plot analysis of two-colour indirect immunofluorescence staining of PBM with IL-A17 (IgG1) and IL-A12 (IgG2a) utilizing isotype-specific biotin-conjugated anti-mouse IgG1/phycoerythrin-avidin (red fluorescence) and FITC-conjugated anti-mouse IgG2a (green fluorescence).

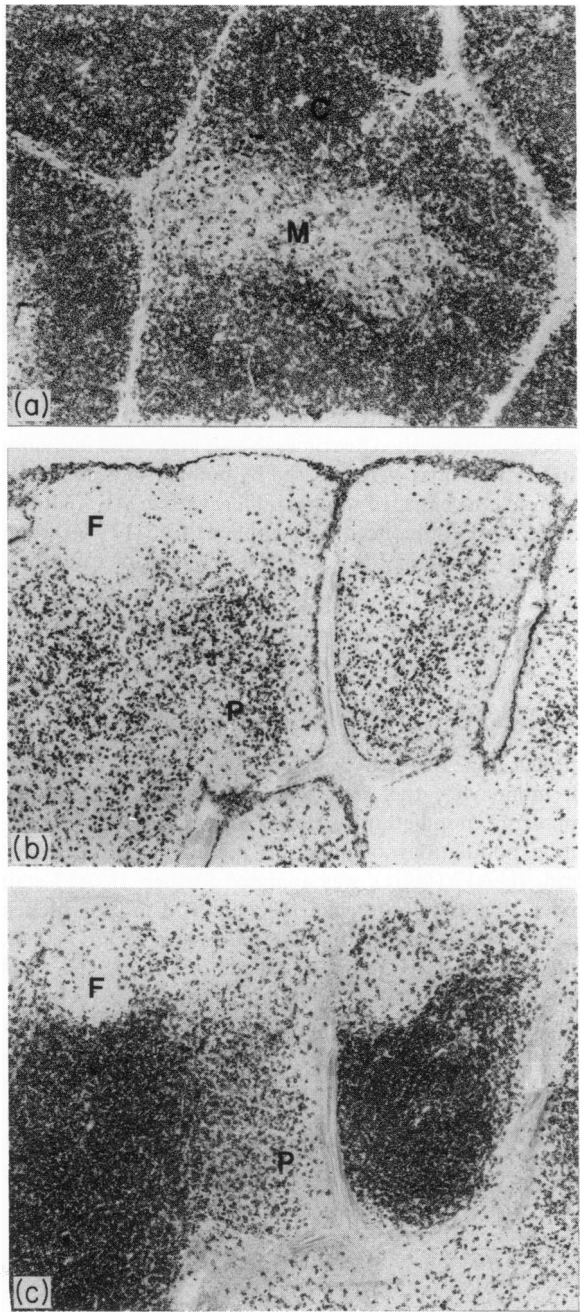

Figure 2. Sections of normal bovine lymphoid tissues stained by the immunoperoxidase technique with IL-A17 and IL-A12. In the thymus (a), the mAb IL-A17 reacts with the majority of cells in the cortex (C) and a subpopulation in the medulla (M). In lymph node (b), IL-A17-positive cells constitute a small subpopulation of cells in the paracortex (P). In comparison, a section of the same lymph node stained with IL-A12 (c) demonstrates a large population of BoT4$^+$ cells in the paracortex (magnification $\times$ 56).

immunoglobulin-positive population. IL-A17 also did not react with granulocytes. IL-A17 stained approximately 70% of cells in cell suspensions of thymus.

Two-colour immunofluorescence was used to determine the relationship of cells stained by IL-A17 with those detected by another mAb, IL-A12, which reacts with a molecule (BoT4) that has a distribution and biochemical and functional characteristics similar to human CD4 (Baldwin *et al.*, 1986; Teale *et al.*, 1986). As indicated in Fig. 1b, dual staining with the two mAbs revealed two distinct populations of T cells. Less than 2% of PBM were positive with both antibodies.

Tissue distribution of IL-A17$^+$ cells

In order to determine the anatomical distribution of IL-A17$^+$ cells, cryostat sections of lymphoid tissue and bone marrow were stained with IL-A17 by the indirect immunoperoxidase technique. Only very small numbers of IL-A17$^+$ cells were found scattered individually throughout the bone marrow (not shown). In the thymus, IL-A17 gave intense staining of the majority of cortical thymocytes and a small population of lymphocytes in the medulla (Fig. 2a). In lymph nodes, IL-A17 stained a subpopulation of lymphocytes in the paracortex and medullary cords and occasional cells within B-cell dependent follicular zones (Fig. 2b). In the spleen (not shown), IL-A17 stained a proportion of lymphocytes within the periarteriolar regions and marginal zones but virtually no cells in the follicular areas. A few IL-A17$^+$ lymphocytes were also discernible within the red pulp.

Analysis of molecules detected by mAb IL-A17

When proteins from lysed IL-A17$^+$ CTL were separated by SDS–PAGE under denaturing and reducing conditions, transferred to nitrocellulose and probed with IL-A17, two faint bands of approximately 34,000 and 35,000 MW were detected (Fig. 3).

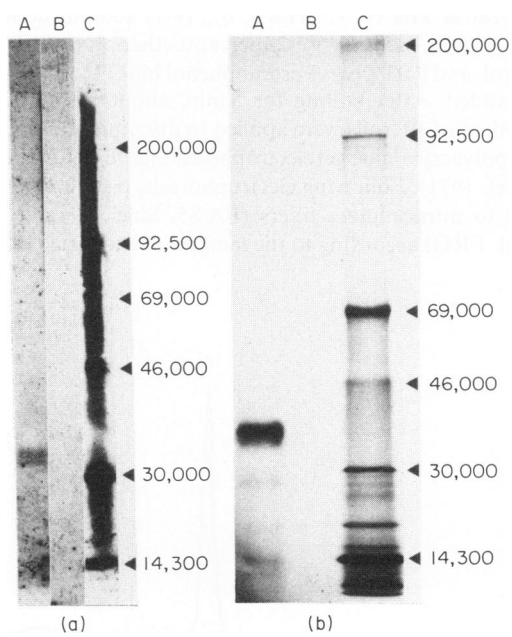

Figure 3. (a) Immunoblot analysis of extracts from cloned IL-A17$^+$ CTL with IL-A17 (Lane A), or an unrelated IgG1 mAb (Lane B). Molecular weight markers are indicated in Lane C. (b) Analysis by SDS–PAGE under reducing conditions of the molecules precipitated from surface-labelled cloned IL-A17$^+$ CTL with IL-A17 (Lane A), or unrelated IgG1 mAb (Lane B). Molecular weight markers are indicated in Lane C.

Table 1. Response of FACS-sorted ILA17$^+$ and ILA17$^-$ PBM to mitogenic and alloantigenic stimulation*

Experiment	Stimulation	TCGF added	PBM population		
			Unsorted	IL-A17$^+$	IL-A17$^-$
I.	Medium	−	205±101	27±26	80±51
	PHA	−	33,949±3879	4888±180	36,232±3683
	Con A	−	30,363±875	913±25	34,494±6700
	Medium	+	7841±228	1003±69	12,174±1750
	PHA	+	35,168±2078	11,280±338	44,089±5467
	Con A	+	30,729±2922	16,535±515	34,479±3953
II.	Medium	−	1049±402	531±539	1318±139
	Allogeneic PBM	−	76,601±10,189	20,539±11,241	90,168±2708
	Medium	+	6756±2119	2515±1896	4517±1846
	Allogeneic PBM	+	99,753±8799	67,503±5917	93,095±5918

* Mean±SD of c.p.m. of incorporated [^{125}I]iododeoxyuridine for cultures of two representative experiments are shown. All cultures shown here were performed in half-area microtitre plates as described previously (Baldwin *et al.*, 1986).

Immunoprecipitation with IL-A17 or surface-labelled molecules from IL-A17$^+$ CTL (Fig. 3) or thymocytes (data not shown), followed by analysis under reducing conditions, revealed a prominent 35,000 MW band and a faint 34,000 MW band in both instances. Under non-reducing conditions, immunoprecipitates from surface-labelled CTL migrated as a broad heavy band at approximately 200,000 MW and faint bands at approximately 140,000 and 70,000 MW (data not shown), suggesting that the precipitated antigen exists as dimers and other multimers.

Responses of IL-A17$^+$ lymphocytes to stimulation with mitogens and alloantigens

The level of proliferation of IL-A17$^+$ cells in response to the mitogens PHA and Con A was profoundly lower (85% reduction) than that for unsorted or IL-A17$^-$ populations (Tables 1 and 2). Addition of exogenous TCGF (Table 1) markedly increased proliferation in the IL-A17$^+$ population; however, in no instance did this equal the level of proliferation in the unsorted and IL-A17$^-$ populations. Addition of 10% autologous monocytes did not increase the response of the IL-A17$^+$ cells in the presence or absence of TCGF (Table 2). Similarly, the IL-A17$^+$ population exhibited a reduced capacity to proliferate in primary allogeneic MLC, although the reduction was less marked than for mitogenic stimulation. Again, exogenous TCGF increased the proliferative capacity of IL-A17$^+$ cells (Table 1).

Ability to inhibit proliferation and cytotoxicity

IL-A17 had no inhibitory effect on proliferation of PBM to mitogens or alloantigens (Table 3), nor any apparent influence on the generation of cytotoxic cells in MLC (data not shown). In contrast, when effector populations from four allogeneic combinations were incubated with IL-A17 for 1 hr prior to testing for cytotoxic activity, cytotoxicity was inhibitied by 0, 28, 56 and 58%, compared to untreated effector cells, at an effector:target ratio of 40:1. The results of one experiment are shown in Fig. 4a.

Table 2. Response of FACS-sorted IL-A17$^+$ and IL-A17$^-$ PBM to mitogenic and alloantigenic stimulation with 10% autologous monocytes added*

Stimulation	TCGF added	PBM population		
		Unsorted	IL-A17$^+$	IL-A17$^-$
Medium	−	202±69	427±146	509±141
Con A	−	14,946±2898	1358±562	9212±1691
Medium	+	1461±292	404±18	1545±99
Con A	+	19,127±2428	6840±2389	13,089±4407

* See footnote for Table 1. 10% monocytes were added to all cultures shown.

Table 3. Effect of IL-A17 on proliferative responses of PBM to mitogenic and alloantigenic stimulation*

Additives to cultures†	Proliferative stimulus		
	PHA	Con A	Alloantigen
None	207,264±21,378	218,624±19,619	61,980±22,008
mAb IL-A17	238,662±96,814	172,379±18,028	58,839±1246
Control mAb‡	156,044±8660	199,274±13,892	41,085±977

* Results indicated are as per Table 1, except that assays were performed in standard microtitre plates.

† mAbs were added at 50–100-fold their endpoint dilution as ascitic fluid to mitogen-stimulated cultures, or as purified Ig to alloantigen-stimulated cultures.

‡ The control mAb added to mitogen-stimulated cultures was IL-A12, an IgG2a, which reacts with BoT4 (Baldwin *et al.*, 1986); that added to alloantigen-stimulated cultures was IL-A5, an IgG1, which recognizes class I determinants on the responder PBM, but not on the stimulator PBM.

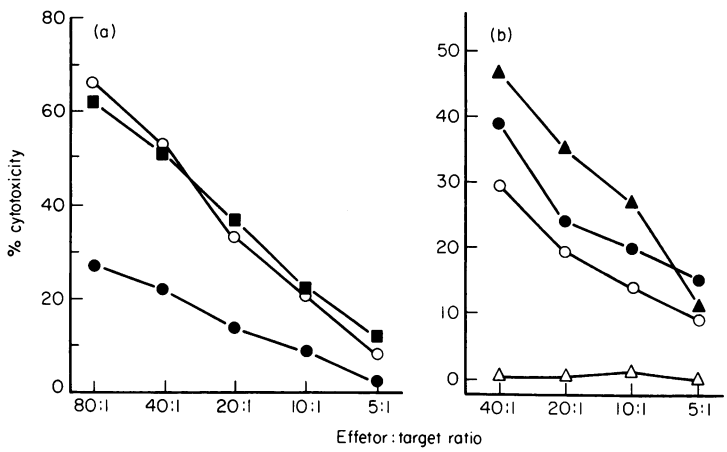

Figure 4. (a) Ability of IL-A17 to block cytotoxicity generated in an allogeneic MLC. Effector cells were tested in a 4-hr ^{51}Cr-release assay on *Theileria parva*-infected lymphoblastoid target cells. Cytotoxicity by effector cells incubated with IL-A17 (●), IL-A12 (○) or no mAb (■) is shown. (b) Cytotoxic activity of IL-A17$^+$ and IL-A17$^-$ cells obtained from an allogeneic MLC established with the same animals as listed in (a). Effector cells were tested in a 4-hr ^{111}In-release assay. Cytotoxic activity of FACS-sorted IL-A17$^+$ (▲) and IL-A17$^-$ (△) populations and unfractionated populations either unstained (●) or stained with IL-A17 (○) is shown.

Cytotoxic activity of IL-A17$^+$ lymphocytes

Sorting of effector cells generated in an MLC into IL-A17$^+$ and IL-A17$^-$ populations and testing on target cells derived from a lymphoblastoid cell line not expressing detectable class II MHC antigens showed that cytotoxic activity resided in the IL-A17$^+$ population (Fig. 4b). There was enrichment for cytotoxic activity in the IL-A17$^+$ cells compared with unsorted populations, both in stained and unstained populations that were subjected to the same washing procedures as sorted cells. All effector populations gave very low levels (less than 5%) of cytotoxicity on the class I MHC mismatched target.

DISCUSSION

Herein we report the production and characterization of a mAb, IL-A17, which recognizes a membrane determinant on a discrete population of bovine T lymphocytes, distinct from those expressing BoT4, defined by mAb IL-A11 and IL-A12 (Baldwin *et al.*, 1986; Teale *et al.*, 1986). On the basis of distribution and immunochemical and functional data, we propose that this antibody defined BoT8, an antigen analogous to human CD8 and similar molecules in other species.

CD8-like molecules are expressed by approximately 20–35% of mature peripheral T lymphocytes, but are present on the majority of cortical thymocytes (Reinherz *et al.*, 1980; Ledbetter *et al.*, 1981; Matsurra *et al.*, 1984; Pescovitz *et al.*, 1985; Maddox *et al.*, 1985). A similar distribution of IL-A17$^+$ cells was found in cattle. In man (Ledbetter *et al.*, 1981), dual expression of CD8 and CD4 molecules occurs on up to 2% of peripheral lymphocytes from normal individuals. A similar level of double positive

cells was detected in bovine PBM, although it could not be determined whether this represented background staining or true dual expression. This is in contrast to the 6–15% level of extrathymic dual expression reported for equivalent molecules in swine (Pescovtiz *et al.*, 1985). As described in other species (Van Ewijk *et al.*, 1981; Poppema *et al.*, 1981; Maddox *et al.*, 1985), lymphocytes expressing BoT8 molecules in cattle comprise less than one half of the population within the T dependent zones in lymph nodes and spleen, and are rarely found in B-dependent zones in lymph nodes and spleen, and follicular areas.

In most species examined, two reduced forms of CD8-like molecules are found on thymocytes, while a single form is expressed on peripheral lymphocytes (Ledbetter *et al.*, 1981; Matsuura *et al.*, 1984; Maddox *et al.*, 1985). However, immunoprecipitation of extracts from swine lymphocytes using mAb directed against CD8-like molecules has demonstrated both 35,000 MW (Pescovitz *et al.*, 1984) and 33,000–35,000 MW (Jonjic & Koszinowski, 1984) reduced molecular forms in the peripheral blood. When analysed under non-reducing conditions, immunoprecipitations with IL-A17 of molecules from surface-labelled bovine thymocytes and cloned CTL demonstrated large molecular weight forms which, under reducing conditions, migrated as 34,000–35,000 MW doublets. Immunoblotting of reduced CTL proteins with IL-A17 revealed two bands of comparable molecular weights, suggesting that the epitope recognized by IL-A17 is shared by two polypeptides. It has been suggested that precipitation of different forms of CD8 on thymic and mature lymphocytes possibly results from variable glycosylation related to maturation (Snow & Terhorst, 1983). Our results detecting two reduced forms on both thymocytes and CTL may relate to the usage of activated cells rather than resting T lymphocytes in immunochemical studies.

Experiments employing highly purified IL-A17$^+$ (96–99%) populations demonstrated that BoT8$^+$ cells respond very poorly to mitogens or alloantigens in the absence of exogenous growth factors. This, therefore, may be attributable to the inability of these cells to produce growth factors, such as interleukin-2, as the addition of exogenous TCGF largely restored the response. Elsewhere we have presented evidence to suggest that BoT4$^+$ cells, which are within the IL-A17$^+$ population, are capable of producing the necessary growth factors (Baldwin *et al.*, 1986). Similarly, cells expressing CD8-like molecules in humans and swine respond less well to PHA when compared with cells expressing CD4-like molecules; reduced responses to alloantigens are also seen with PT8$^+$ swine cells (Reinherz *et al.*, 1979, 1980; Pescovitz *et al.*, 1985). By contrast, CD8$^+$ cells in humans respond almost as well to allostimulation and Con A (Reinherz *et al.*, 1980) as do the CD4$^+$ cells (Reinherz *et al.*, 1979), and Ly 2,3$^+$ cells in mice respond to Con A as well as do the Ly 1$^+$ cells (Hollander, 1982; Jadinski *et al.*, 1976).

The failure of IL-A17 to block mitogen and alloantigen-stimulated proliferation is consistent with that reported in other species for mAb directed against CD8-like molecules (Reinherz *et al.*, 1980; Pescovitz *et al.*, 1985). There was marked variation in the level of blocking by IL-A17 of allospecific cytotoxicity obtained with different effector/target combinations. There are a number of possible explanations for this, which include differences between individuals in qualitative and quantitative clonal responses to class I and class II MHC antigens and/or the avidity of the effector cells for the target cell. These, in turn, may have been influenced by different degrees of disparity in MHC combinations. Evidence for the importance of some of these factors has been presented in studies employing CTL clones in other species (Meuer, Schlossman & Reinherz, 1982; MacDonald, Glasbrook & Cerottini, 1982; Platsoucas, 1984). Mechanisms contributing to the variable ability of IL-A17 to block function of bovine CTL have been further dissected at the clonal level in another report (Teale *et al.*, 1986). Contrary to the ability of IL-A17 to at least partially block the cytolytic event in some experiments, there was no apparent blocking of generation of effector cells. However, the possibility that some clonal responses were reduced or ablated cannot be excluded.

We have shown in one experiment with CTL generated in an allogeneic MLC that cytotoxic effector function resided within the BoT8$^+$ population. The target cell used in this experiment did not express detectable levels of class II MHC antigens, suggesting that the effector function measured was specific for class I MHC products. Furthermore, recent observations on cloned BoT8$^+$ alloreactive CTL clones have demonstrated their specificity for class I MHC determinants (Teale *et al.*, 1986). Together with the above characteristics, these results are consistent with the notion that IL-A17 defines a CD8 analogue on bovine T lymphocytes.

ACKNOWLEDGMENTS

The authors acknowledge E. Awino, P. Belsoi, D. Ngugi and G. Lamb for expert technical assistance, and J. Magondu and D. Wambuthi for assistance with FACS analyses. We also wish to thank Dr W. C. Davis, Washington State University, for advice and assistance with dual colour immunofluorescence. This is ILRAD publication No. 449.

REFERENCES

BALDWIN C.L., TEALE A.J., NAESSENS J., GODDEERIS B.M., MACHUGH N.D. & MORRISON W.I. (1986) Characterization of a subset of bovine T-lymphocytes expressing B0T4 by monoclonal antibodies and function: similarity to lymphocytes defined by human T4 and murine L3T4. *J. Immunol.* (in press).

BURNETTE W.N. (1981) 'Western blotting': electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein. *Analyt. Biochem.* **112,** 195.

GODDEERIS B.M., BALDWIN C.L., OLE-MOI YOI O. & MORRISON W.I. (1986) Improved methods for purification and depletion of monocytes from bovine peripheral blood mononuclear cells. Functional evaluation of monocytes in response to lectins. *J. immunol. Meth.* (in press).

HOLLANDER N. (1982) Effects of anti-Lyt antibodies on T cell functions. *Immunol. Rev.* **68,** 43.

IUIS-WHO NOMENCLATURE SUBCOMMITTEES (1984) Nomenclature for clusters of differentiation (CD) of antigens defined by human leukocyte populations. *Bull. W.H.O.* **62,** 809.

JADINSKI J., CANTOR H., TADAKUMA J., PEAVY D.L. & PIERCE C.W. (1976) Separation of helper T cells from suppressor T cells expressing different LY components. Polyclonal activation: suppression and helper activities are inherent properties of distinct T cell subclasses. *J. exp. Med.* **143,** 1382.

JONJIC S. & KOSZINOWSKI U.H. (1984) Monoclonal antibodies reactive with swine lymphocytes. I. Antibodies to membrane structures that define the cytolytic T lymphocyte subset in the swine. *J. Immunol.* **133,** 647.

LALOR P.A., MORRISON W.I., GODDEERIS B.M., JACK R.J. & BLACK S.J. (1986) Monoclonal antibodies identify phenotypically and functio-

nally distinct cell types in the bovine lymphoid system. *Vet. Immunol. Immunopath.* (in press).

LANIER L.L., ENGELMAN E.G., GATENBY P., BABCOCK G.F., WARNER N.L. & HERZENBERG L.A. (1983) Correlation of functional properties of human lymphoid cell subsets and surface marker phenotypes using multiparameter analysis and flow cytometery. *Immun. Rev.* **74,** 143.

LEDBETTER J.A., EVANS R.L., LIPINSKI M., CUNNINGHAM-RUNDLES C., GOOD R.A. & HERZENBERG L.A. (1981) Evolutionary conservation of surface molecules that distinguish T lymphocyte helper/inducer and cytotoxic subpopulations in mouse and man. *J. exp. med.* **153,** 310.

MACDONALD H.R., GLASEBROOK A.L. & CEROTTINI J.C. (1982). Clonal heterogeneity in the functional requirement for LY-2/3 molecules on cytolytic lymphocytes: analysis by antibody blocking and selective trypsinization. *J. exp. Med.* **156,** 1711.

MADDOX J.F., MACKAY C.R. & BRANDON M.R. (1985) Surface antigens, SBU-T4 and SBU-T8, of sheep T lymphocyte subsets defined by monoclonal antibodies. *Immunology*, **55,** 739.

MAIZEL J.V. (1971) Polyacrylamide gel electrophoresis of viral proteins. *Meth. Virol.* **5,** 179.

MATSUURA A., ISHII Y., YUASA H., NARITA H., KON S., TAKAMI T. & KIKUCHI K. (1984) Rat T lymphocyte antigens comparable with mouse Lyt-1 and Lyt-2, 3 antigenic systems: characterization by monoclonal antibodies. *J. Immunol.* **132,** 316.

MEUER S.C., SCHLOSSMAN S.F. & REINHERZ E.L. (1982 Clonal analysis of human cytotoxic T lymphocytes: $T4^+$ and $T8^+$ effector T cells recognize products of different major histocompatibility complex antigens. *Proc. natl. Acad. Sci. U.S.A.* **79,** 4395.

PEARSON T.W., PINDER M., ROELANTS G.E., KAR S.K., LUNDIN L.B., WITHEY K.S. & HEWETT R.S. (1980) Methods for derivation and detection of anti-parasite monoclonal antibodies. *J. immunol. Meth.* **34,** 141.

PESCOVITZ M.D., LUNNEY J.K. & SACHS D.H. (1984) Preparation and characterization of monoclonal antibodies reactive with porcine PBL. *J. Immunol.* **133,** 368.

PESCOVITZ M.D., LUNNEY J.K. & SACHS D.H. (1985) Murine anti-swine T4 and T8 monoclonal antibodies: distribution and effects on proliferative and cytotoxic T cells. *J. Immunol.* **134,** 37.

PLATSOUCAS C.D. (1984) Human T cell antigens involved in cytotoxicity against allogeneic or autologous chemically modified targets. Association of the Leu 2a/T8 antigen with effector-target cell binding and of the T3/Leu 4 antigen with triggering. *Eur. J. Immunol.* **14,** 566.

POPPEMA S., BHAN A.K., REINHERZ E.L., MCCLUSKEY R.T. SCHLOSSMAN S.F. (1981) Distributuion of T cell subsets in human lymph nodes. *J. exp. Med.* **153,** 30.

REINHERZ E.L., KUNG P.C., GOLDSTEIN G. & SCHLOSSMAN S.F. (1979) Separation of functional subsets of human T cells by a monoclonal antibody. *Proc. natl. Acad. Sci. U.S.A.* **76,** 4061.

REINHERZ E.L., KUNG P.C., GOLDSTEIN G. & SCHLOSSMAN S.F. (1980) A monoclonal antibody reactive with the human cytotoxic/suppressor T cell subset previously defined by a heteroantiserum termed TH. *J. Immunol.* **129,** 1301.

SHAPIRO S.Z., VOIGT W.P. & FUJISAKI K. (1986) Tick antigens recognized by serum from a guinea pig resistant to infestation with the tick *Rhipicephalus appendiculatus*. *J. Parasit.* (in press).

SHORTMAN K. & WILSON A. (1981) A new assay for cytotoxic lymphocytes, based on a radioautographic readout of ^{111} In release, suitable for rapid, semi-automated assessment of limiting dilution cultures. *J. immunol. Meth.* **43,** 135.

SNOW P.M. & TERHORST C. (1983) The T8 antigen is a multimeric complex of two distinct subunits on human thymocytes but consists of homomultimeric forms on peripheral blood T lymphocytes. *J. biol. Chem.* **258,** 14675.

STRAUS W. (1982) Imidazole increases the sensitivity of the cytochemical reaction for peroxidase and diaminobenzidine at neutral pH. *J. Histochem. Cytochem.* **30,** 491.

SWAIN S.L. (1983) T cell subsets and the recognition of MHC class. *Immunol. Rev.* **74,** 129.

TEALE A.J., BALDWIN C.L., ELLIS J.A., NEWSON J., MORRISON W.I. & GODDEERIS B.M. (1986) Alloreactive bovine T cell clones: an analysis of function, phenotype and specificity. *J. Immunol.* (in press).

TEALE A.J., MORRISON W.I., GODDEERIS B.M., GROOCOCK C.M., STAGG D.A. & SPOONER R.L. (1985) Bovine alloreactive cytotoxic T cells generated *in vitro*: target specificity in relation to BoLA phenotype. *Immunology*. **55,** 355.

VAN EWIJK W., VAN SOEST P.L. & VAN DEN ENGH J. (1981) Fluorescence analysis and anatomic distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2 and T-200. *J. Immunol.* **127,** 2594.