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Bovine T lymphocytes

I. GENERATION AND MAINTENANCE OF AN INTERLEUKIN-2-DEPENDENT, CYTOTOXIC T-LYMPHOCYTE CELL LINE

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SUMMARY

Primary and secondary bovine allogeneic mixed leucocyte cultures were examined for the generation of antigen-specific cytotoxic leucocytes. While optimal generation of murine and human cytotoxic T lymphocytes typically requires 4–8 days, alloantigen-specific cytotoxic bovine leucocytes were demonstrated consistently only after prolonged incubation periods, optimally found to be about 15 days. Restimulation of long-term bovine mixed leucocyte cultures with the original stimulator population revealed responder cells demonstrating augmented alloantigen-specific lytic activity. When placed into human recombinant interleukin-2, responder cells expanded and required passaging every 3–4 days. The same was not true of cells placed into interleukin-2-free medium. Cells cultured in interleukin-2-containing medium retained alloantigen specificity after 10 weeks of culture. Moreover, they continued to display total dependence on human, simian or bovine interleukin-2 for growth.

INTRODUCTION

Studies examining cell-mediated immunity in cattle have been hampered by a paucity of suitable monospecific reagents to differentiate between the cellular elements involved in immune responses. While investigators agree that bovine B lymphocytes are those cells which express surface immunoglobulin, the identification of bovine T cells has not been as simple. For example, numerous investigators have reported that those bovine lymphocytes which rosette with sheep red blood cells are of the T-cell lineage (Buschmann & Pawlas, 1980; Beldon, McCroskey-Rothwell & Strelkauskas, 1981). Others have claimed that bovine T cells respond to lectins, such as concanavalin A (Con A) and phytohaemagglutinin, which are known to induce blastogenesis of murine and human T cells (Beldon & Strelkauskas, 1981; Brownlie & Stott, 1979). More recently, monoclonal antibodies (MoAb) reactive with bovine leucocytes and specific leucocyte subsets have been purported (Davis, Perryman & McGuire, 1984). These MoAb have typically been generated from murine myelomas fused with splenocytes of mice hyperimmunized with multiple species of xenogeneic leucocytes. In theory, antibody to phylogenetically conserved regions of leucocyte surface antigens would be cross-reactive between disparate animal species. Therefore, anti-murine killer T-cell specific antibody could potentially bind to bovine killer T cells, but not to helper cells.

Rather than define a subpopulation of bovine leucocytes based upon staining patterns, response to lectins, or the like, we chose to use a physiological marker, antigen-specific cytotoxicity. We found that alloantigen-specific cytotoxic bovine leucocytes would grow, apparently indefinitely, in the presence of human recombinant interleukin-2 (rIL-2), lectin-free simian interleukin-2 (IL-2)-containing supernatant fluid, as well as bovine lymph node (LN)-conditioned medium (CM). They would not grow in medium alone. Finally, we found that the cells retained antigen specificity for at least 10 weeks in culture, while simultaneously incorporating tritiated thymidine in a strict IL-2 dose-dependent fashion. Based upon these observations, we have concluded they are IL-2-responsive, antigen-specific bovine T lymphocytes.

Abbreviations: BT2, bovine cytotoxic T-cell line; CM, conditioned medium; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; E:T, effector to target cell ratio; FBS, fetal bovine serum; IL-2, interleukin-2; IMDM, Iscove's medium; LMC, lymphocyte-mediated cytolysis; LN, lymph node; LPS, lipopolysaccharide; MLC, mixed leucocyte culture; MoAb, monoclonal antibody; PBL, peripheral blood leucocytes; rIL-2, recombinant interleukin-2; TCGF, T-cell growth factor.

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MATERIALS AND METHODS

Medium

Supplemented RPMI-1640 medium (Cat. no. 430-1800, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) and Iscove's serum-free medium (IMDM) were prepared as described in a previous paper (Baker & Knoblock, 1982a). KC-

2000 medium, an experimental serum-free medium, was generously provided by KC Biologicals, Lenexa, KS.

Primary mixed leucocyte culture (MLC)

Bovine peripheral blood leucocytes (PBL) were from 2-year-old Hereford steers maintained at the Diamond Shamrock research farm, Painesville, OH. The PBL from these cattle were harvested and mononuclear cells isolated as previously described (Baker & Knoblock, 1982a) and adjusted to $2{\cdot}5 \times 10^6$ cells/ml in KC-2000 medium with 25 μg/ml *E. coli* lipopolysaccharide (LPS) (Cat. no. 3120-25, Difco, Detroit, MI). Aliquots of 25 ml were placed in 75 cm^2 culture flasks (Cat. no. 25110, Corning, Corning, NY) and cultured for 68–72 hr at 37 in a humidified atmosphere of 5% CO_2 in air. After harvesting by centrifugation, the cell pellet was resuspended in RPMI-1640 medium and inactivated with mitomycin *c* (Cat. no. M0503, Sigma, St Louis, MO) at 30 μg/ml for 1 hr. This suspension was washed twice through 10 ml FBS and adjusted to 2×10^6 cells/ml in supplemented RPMI-1640 medium.

Responder cells in the MLC were fresh PBL, prepared as described above, and adjusted to 2×10^6 cells/ml in supplemented RPMI-1640 medium. Ten ml each of the responder and stimulator cell populations were cultured upright in cell culture flasks (Cat. no. 3012, Falcon, Division of Becton-Dickinson, Oxnard, CA).

Secondary mixed leukocyte culture

Responder cells were harvested from primary MLC at the times indicated, and viable cells were recovered by centrifugation through Ficoll–Hypaque (Histopaque, Cat. no. 1077–1, Sigma). The cells were washed twice in PBS and adjusted to 2×10^6 cells/ml in supplemented RPMI-1640 medium.

Stimulator cells were mitomycin *c*-inactivated LPS blasts, prepared as described above, and adjusted to 2×10^6 cells/ml in supplemented RPMI-1640 medium. One-ml aliquots of the responder and stimulator cell populations were added to 24-well cluster plates (Cat. no. 3524, Costar, Cambridge, MA). Alternatively, equal volumes (10 ml) of responder and stimulator cells were added to culture flasks (Cat. no. 3012, Falcon) incubated upright. In either case, the secondary MLC were incubated at 37 in a humidified atmosphere of 5% CO_2 in air for indicated times.

Lymphocyte-mediated cytotoxicity (LMC) assay

All LMC assays were performed by the method described by Gillis & Smith (1977a), with minor modifications. Effector cells were harvested from primary or secondary MLC at the times indicated and viability was determined by microscopic observation of trypan blue exclusion. Cells were resuspended in supplemented RPMI-1640 medium and the cell concentration was determined. Log_2 dilutions were made, and 100-μl aliquots of each dilution were added to triplicate wells of conical microtitre plates (Cat. no. 76-042-05, Linbro, Division of Flow Laboratories, McLean, VA).

Target cells were prepared by culturing fresh bovine PBL in 75-cm^2 flasks (Corning) at 1×10^6 cells/ml in IMDM medium with 0·31 μg/ml Con A (Cat. no. 79-003, Miles-Yeda, Rehovot, Israel) for 48 hr. Viable Con A blasts were harvested by centrifugation over Histopaque, and labelled with 350 μCi of ^{51}chromium [^{51}Cr, as sodium chromate, Cat. no. NEZ-030S, New England Nuclear (NEN), Boston, MA]. Cells were washed and resuspended in supplemented RPMI-1640 medium, and adjusted to give an effector:target cell ratio (E:T) of at least 100:1. Aliquots of 100 μl were added to the microtitre plates already containing the effector cells. The plates were centrifuged at 200 *g* for 10 min at room temperature and incubated in a humidified atmosphere of 5% CO_2 in air at 37 for 4 hr.

The ^{51}Cr-release reaction was stopped by a 350 *g* centrifugation at 4 for 10 min. A 100-μl supernatant sample from each well of the triplicate cultures was added to 3·5 ml Biofluor Scintillant (Cat. no. NEF-961, NEN) and counted on a liquid scintillation counter serially connected to a microcomputer. Data reduction was accomplished using a BASIC program to compute the means and standard deviations of stored data (Roche & Baker, 1983).

Spontaneous release was determined by incubating triplicate cultures of target cells with medium only, and maximum release was found by incubating target cells with a detergent solution (Cat. no. B3157-15, Scientific Products, McGraw Park, IL). Percentage specific lysis was determined using the following formula:

$$\%\ \text{specific lysis} = \frac{\text{mean sample c.p.m.} - \text{mean spontaneous c.p.m.}}{\text{mean maximum c.p.m.} - \text{mean spontaneous c.p.m.}} \times 100\%$$

where c.p.m. represents counts per minute.

Interleukin-2

MLA-144, a constituitive IL-2-producing primate cell line, was generously provided by Dr Gary Splitter, University of Wisconsin, Madison. MLA-144 cells were seeded at 1×10^6/ml in supplemented RPMI-1640 medium and incubated at 37 for 24 hr in a humidified atmosphere of 5% CO_2 in air. Cells were removed by centrifugation, and the supernatant fluid was filtered through a 0·45 μm filter. The MLA-144 CM could be stored at 4 for several months without noticeable loss of activity.

Recombinant-derived human IL-2 was obtained as the result of a collaborative research project between Immunex Corporation and Hoffman–La Roche, Nutley, NJ. This rIL-2 had a specific activity of 1×10^3 units/μg when assayed on bovine IL-2-responsive leucocytes.

A cell suspension was prepared from bovine retropharyngeal LN. The suspension was washed and resuspended in supplemented RPMI-1640 medium containing 7·5 μg/ml Con A. Flasks containing the lectin-activated LN cells were incubated for 24 hr at 37 in a humidified atmosphere of 5% CO_2 in air. Cells were removed by centrifugation and the supernatant fluid was filtered through a 0·45-μm filter. Bovine LN CM were stored at 4 until use.

All preparations of IL-2 utilized were quantified for lymphokine activity utilizing a bovine IL-2-responsive cell line (Baker & Knoblock, 1982b). Determination of specific activity was performed in a micro-IL-2 assay similar to that described by Gillis *et al.* (1978b). Radionucleotide incorporation was determined utilizing a liquid scintillation counter serially connected to a microcomputer, as described earlier. Interleukin-2 activity was determined with the aid of a computer program employing iterative weighting of individual samples linearized by logit transformation (Davis *et al.*, 1983). A defined 1 unit/ml standard consisted of frozen aliquots of 20% (v/v) MLA-144 CM.

RESULTS

Cytotoxic response in primary culture

Viable effector cells from short-term (4–7 days) primary MLC were examined for cytotoxic activity on various days in three separate experiments. Target cell populations tested in LMC assays included: (1) allogeneic Con A-induced lymphoblast cells from the same animal as the original stimulating population; (ii) autologous Con A lymphoblast cells from the responding animal; and, (iii) third-party Con A lymphoblast cells from an animal not utilized in the original primary MLC. Maximum cytolysis observed in all experiments occurred on Day 5 (Table 1). However, significant lysis of alloantigen-specific target cells was not a consistent feature, regardless of the E:T examined.

Since the cytotoxic responses observed in short-term primary MLC were not reproducible, LMC assays were performed on Days 5, 10, 15 and 20 after initiation of primary culture to determine the extent of lytic activity in long-term culture. Alloantigen-specific cytolysis was found to peak on Day 15 (Fig. 1) at approximately 50% lysis (144 E:T). Effectors thus generated demonstrated antigen specificity as seen by the low cytolysis of both autologous and third-party target cells as compared to allogeneic target cells. In addition, there was a distinct E:T dose response of cytolysis.

Cytotoxic response in secondary culture

The lytic activity of the responding population was also determined at various times after secondary restimulation of primary long-term MLC with alloantigen, and was found to peak on Day 5 after restimulation. The effector cell population showed approximately 97% lysis of the allogeneic target cells at the highest E:T tested (Fig. 2). The effector cells also exhibited alloantigen specificity as seen by the minimal cytotoxic activity against the autologous and third-party target cells. In addition, there was a strict dose-dependent relationship between the E:T and percentage specific lysis of the allogeneic target cells.

Table 1. Maximum percentage cytolysis (% cyto) of target cells observed at the highest effector to target cell ratio (E:T) on Day 5 of primary MLC

	Effector cells versus:					
	Allogeneic target cells		Autologous target cells		Third-party target cells	
Exp.	E:T	% cyto	E:T	% cyto	E:T	% cyto
1	161	20	168	10	165	9
2	110	54	129	20	105	4
3	98	5	107	0	101	1

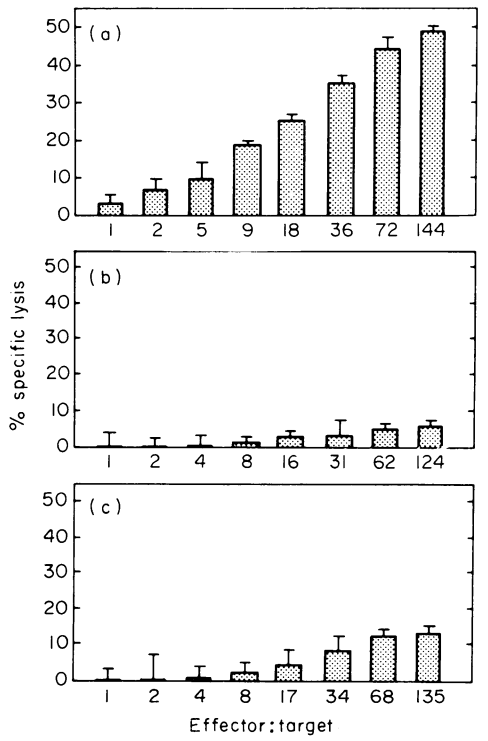

Figure 1. Responding cells were harvested from primary mixed leucocyte culture on Day 15 and tested in a 4-hr ^{51}Cr-release assay against (a) allogeneic lymphoblasts, (b) autologous lymphoblasts, and (c) third-party lymphoblasts. Bars represent the percentage standard deviation of triplicate cultures.

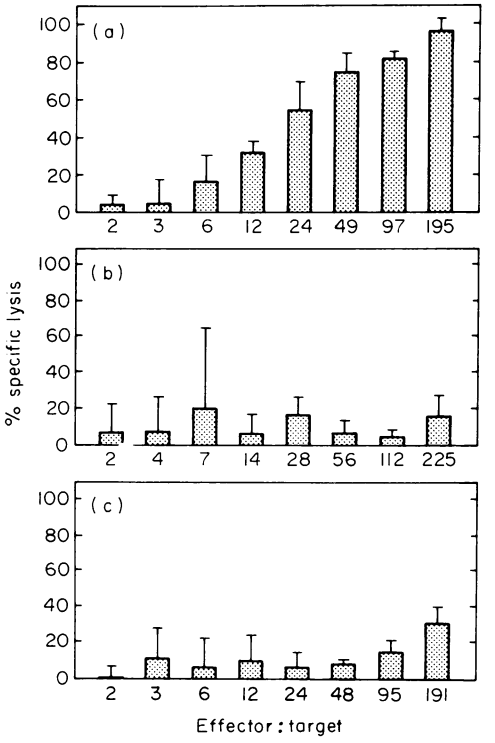

Figure 2. Responding cells were harvested 5 days after restimulation of a long-term primary mixed leucocyte culture with allogeneic leucocytes, and lytic activity determined in a 4-hr ^{51}Cr-release assay against (a) allogeneic lymphoblasts, (b) autologous lymphoblasts, and (c) third-party lymphoblasts. Bars represent the percentage standard deviation of triplicate cultures.

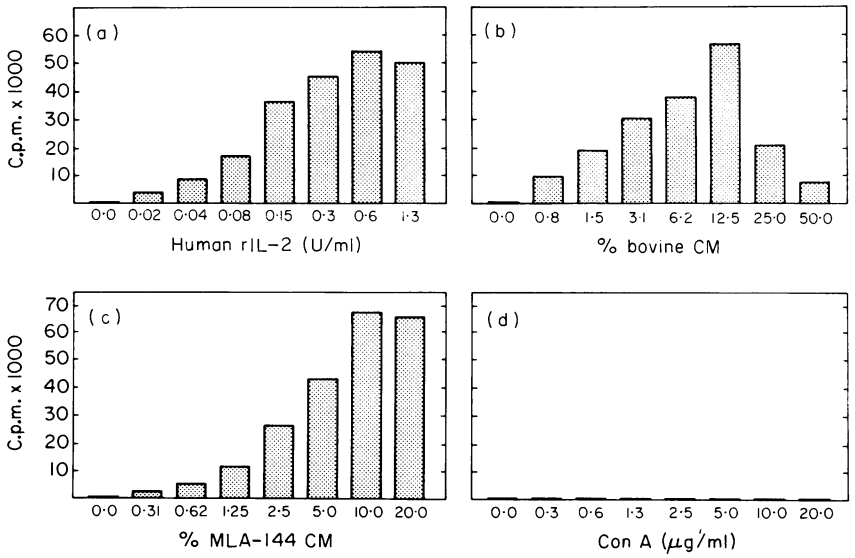

Figure 3. BT2 cells were examined 18 days after initiation for proliferation via [^{3}H]TdR incorporation in response to increasing $\log_2$ dilutions of (a) human recombinant IL-2, (b) bovine lymph node-conditioned medium, (c) MLA-144-conditioned medium, and (d) Con A.

Culture of cytotoxic lymphocytes

IL-2 responsiveness

Alloantigen-specific cytotoxic cells from a secondary MLC population were placed into medium containing 500 pg/ml human rIL-2. Eighteen days after the initiation of culture, these cells (termed BT2) were examined for proliferative responsiveness to various sources of IL-2 or lectin. As determined by [^{3}H]TdR incorporation, BT2 displayed exquisite sensitivity to all sources of IL-2 examined, but no responsiveness to lectin alone (Fig. 3).

Antigen-specific killing

Four weeks after having been placed into IL-2-containing medium, BT2 were assayed for lytic activity against autologous and allogeneic (identical to original stimulators) lymphoblasts

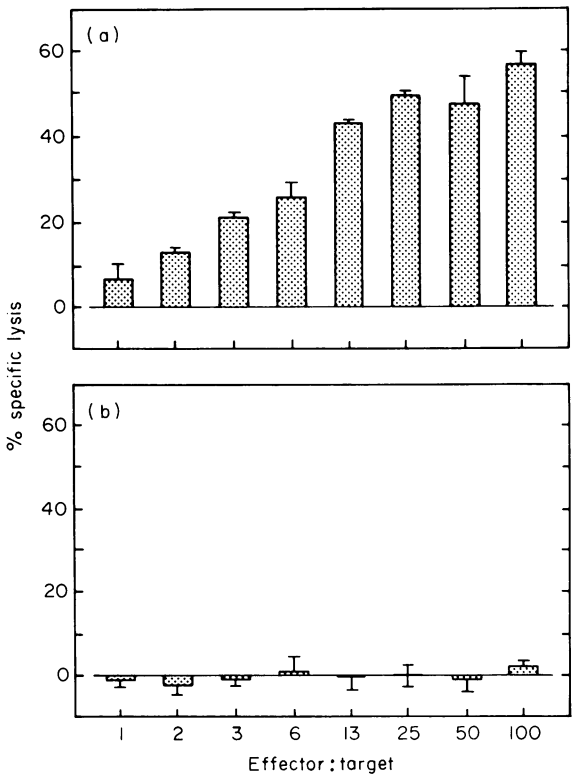

Figure 4. BT2 cells were examined 4 weeks after initiation of culture in a 4-hr ^{51}Cr-release assay for specific cytolysis against (a) allogeneic lymphoblasts and (b) autologous lymphoblasts. Bars represent the percentage standard deviation of triplicate cultures.

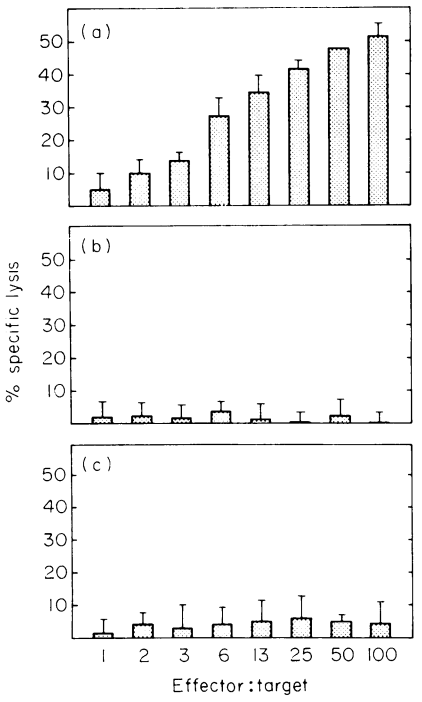

Figure 5. Ten weeks after initiation of culture, BT2 cells were examined for lytic activity in a 4-hr ^{51}Cr-assay against (a) allogeneic lymphoblasts, (b) autologous lymphoblasts, and (c) third-party lymphoblasts. Bars represent the percentage standard deviation of triplicate cultures.

in a 4-hr LMC assay. BT2 effectors displayed approximately 55% specific cytolysis against allogeneic target cells at an E:T of 100:1, while simultaneously showing less than 3% cytotoxicity at the same E:T against autologous lymphoblasts (Fig. 4). Moreover, there was a strict relationship between E:T and percentage cytotoxicity against allogeneic targets, and none against autologous lymphoblasts.

In an effort to determine the effect of extended culturing of BT2 on antigen-specific cytolysis, a similar LMC assay was performed 10 weeks after the initiation of IL-2-dependent culture. In this assay, however, third-party cytolysis was also examined (Fig. 5). Again, BT2 manifested over 50% cytolysis against allogeneic lymphoblasts, and none against autologous cells at an E:T of 100:1. Similarly, there was no significant lysis of third-party lymphoblasts.

DISCUSSION

Our results indicate that bovine primary allogeneic MLC did not generate consistent alloantigen-specific cytotoxic leucocytes in short-term culture. Cytotoxic cells could only be demonstrated on Day 5 in one of three experiments; however, even then there was considerable non-specificity (54% cytolysis against allogeneic targets versus 20% cytolysis against autologous targets at an equivalent E:T) (Table 1). In murine primary MLC, peak cytotoxicity can consistently be observed on Days 4–6 (Wagner & Feldman, 1972; Cerottini *et al.*, 1974), whereas maximum lysis occurs on Days 6–7 in human primary cultures (Sondel & Bach, 1975; Gillis *et al.*, 1978a).

The reasons why the cytotoxic response in bovine primary short-term culture were not reproducible remained unclear. However, it has been shown that adverse environmental or management conditions may cause reduced immune responses in cattle (Blecha & Minocha, 1983; Kelley *et al.*, 1982). The cattle used in the present study were maintained either on pasture or in pens, depending upon weather conditions. It is possible that the conditions under which the animals were kept may have been partially responsible for the observed non-reproducibility.

Due to the inconsistencies seen in short-term primary MLC, cytotoxic activity was examined at Days 5, 10, 15 and 20 after culture initiation to determine the span over which cytolytic leucocytes could be observed in primary long-term MLC. The increased cytotoxicity observed over time was probably due to an increased number of cells that had differentiated into functionally mature cytotoxic T lymphocytes (CTL). MacDonald *et al.* (1975) have reported that functional activation of CTL occurred in the absence of cellular proliferation. In their hands, CTL activity could be generated in murine long-term primary MLC upon re-exposure to alloantigen, even in the presence of a potent inhibitor of DNA synthesis.

Other investigators have determined that viable cells in murine primary long-term MLC were responsive to exogenous sources of IL-2. Baker *et al.* (1978) reported that the addition of supplemental T-cell growth factor (TCGF), now termed IL-2, to mixed tumour-lymphocyte culture generated greater numbers of antigen-reactive effector cells. These effector cells also exhibited augmented cytotoxic activity. Others have found similar results upon the addition of supernatant fluids from secondary MLC to primary long-term MLC cells (Ryser, Cerottini & Brunner, 1978).

In an attempt to expand the population of alloantigen-specific cytotoxic cells, viable cells from long-term cultures were harvested, restimulated with alloantigen and examined for lytic activity. The results of the secondary MLC (Fig. 2) revealed an anamnestic response. The peak lytic activity observed during primary culture approached 50% specific lysis at an E:T of approximately 100:1 on Day 15 (Fig. 1), whereas 5 days after secondary allogeneic restimulation (Fig. 2) the effector cells exhibited over 95% specific lysis of allogeneic target cells at a similar E:T.

Interleukin-2 has been shown to be a requirement for the generation and maintenance of a bovine costimulator-dependent bovine lymphoblastoid cell line (Baker & Knoblock, 1982b), murine cytolytic T-cell lines (Gillis & Smith, 1977b; Baker, Gillis & Smith, 1979; Gillis *et al.*, 1979), and human cytotoxic T-cell lines (Gillis *et al.*, 1978a; Ruscetti, Morgan & Gallo, 1977; Strausser & Rosenberg, 1978). In the present study, bovine alloantigen-specific cytotoxic leucocytes from a secondary MLC were placed into medium containing bovine or primate CM, both known sources of IL-2, or human rIL-2 in an attempt to generate a bovine cytotoxic leucocyte line.

For as long as 10 weeeks after culture initiation, these bovine secondary cytotoxic leucocytes, BT2, displayed specific lysis (Fig. 5), with no decrease in cytotoxic activity compared to that seen at 4 weeks of culture (Fig. 4). Based upon their consistent antigen-specific cytotoxicity, and the observation that these cells not only required IL-2 in order to proliferate, but did so in a dose-dependent fashion with human rIL-2, we must conclude that BT2 is an IL-2-dependent bovine T-cell line.

Cytotoxic T lymphocytes have been found to play a predominant role in a variety of immune reactions. The present study is the first report to demonstrate the *in vitro* generation of alloantigen-specific bovine cytotoxic T lymphocytes. Studies can now be undertaken to determine the steps involved and the agents required for bovine CTL-mediated lysis of target cell populations. In addition, BT2 may be utilized to generate antisera that are reactive with cell surface antigens specific for bovine CTL, all bovine T lymphocytes, or membrane antigens involved in the lytic process, providing monospecific tools for the further study of bovine T cells and T-cell mediated lympholysis. Finally, since we now have a source of relatively homogeneous bovine T cells, we can accurately determine lymphocyte subset specificity of species cross-reactive MoAb.

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