Primary and secondary in vitro generation of bovine cytotoxic T lymphocytes
by Kathleen Elizabeth Senta

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Science
Montana State University

Abstract:
Bovine mixed leukocyte cultures (MLC), involving bovine peripheral blood leukocytes as the responding population and mitomycin-C-inactivated lipopolysaccharide-induced allogeneic leukocyte blast cells as the stimulating population, were examined for in vitro generation of bovine cytotoxic T lymphocytes. Antigen-specific cytotoxic activity was found in primary culture after both short-term and long-term incubation periods. However, significant lysis of allogeneic target cell populations following . short-term primary MLC could not consistently be reproduced. Therefore, secondary allogeneic restimulation of . viable cells from primary MLC was attempted in order to generate effector cells with consistent lytic activity.

Both the proliferative and cytotoxic responses in secondary culture were determined to be anamnestic, reproducible, and alloantigen specific. In addition, it was found that the source of cells used to restimulate long-term responder cells was inconsequential, since both autologous and allogeneic stimulator cells worked equally well. Cells from long-term primary and secondary cultures were shown to be responsive to bovine or primate conditioned medium, containing interleukin 2 (IL2), in a dose-dependent manner. Bovine secondary cytotoxic lymphocytes (SCL) were placed in IL2-containing medium in an attempt to generate an IL2-dependent bovine T lymphocyte cell line. SCL retained their alloantigen lytic specificity after two weeks in culture, and are presently still being maintained in IL2-containing medium. Monoclonal antibody B29B, a 'pan' anti-bovine T cell antibody, was shown cause approximately 45% lysis of radiolabelled-bovine SCL in complement-mediated antibody-dependent cytotoxicity assays. The in vitro generation of bovine cytotoxic T lymphocytes has applications in the future examination of the mechanism of bovine CTL-mediated lympholysis. IL2-dependent bovine SCL may be utilized for the generation of antisera specific for cell surface antigens found on bovine CTL, or all bovine T lymphocytes in general, thereby allowing for definitive identification of bovine T cells. Additionally, this in vitro allogeneic mixed leukocyte culture system can be used to study the role of the bovine major histocompatibility complex in allograft responses and may subsequently prove to be useful for the typing of tissues for organ transplantation.

PRIMARY AND SECONDARY <u>IN VITRO</u> GENERATION OF BOVINE CYTOTOXIC T LYMPHOCYTES

by

Kathleen Elizabeth Senta

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ABSTRACT

Bovine mixed leukocyte cultures (MLC), involving bovine peripheral blood leukocytes as the responding population and mitomycin-C-inactivated lipopolysaccharide-induced allogeneic leukocyte blast cells as the stimulating population, were examined for in vitro generation of bovine cytotoxic T lymphocytes. Antigen-specific cytotoxic activity was found in primary culture after both short-term and long-term incubation periods. However, significant lysis of allogeneic target cell populations following short-term primary MLC could not consistently be reproduced. Therefore, secondary allogeneic restimulation of viable cells from primary MLC was attempted in order to generate effector cells with consistent lytic activity. Both the proliferative and cytotoxic responses in secondary culture were determined to be anamnestic, reproducible, and alloantigen specific. In addition, it was found that the source of cells used to restimulate long-term responder cells was inconsequential, since both autologous and allogeneic stimulator cells worked equally well. Cells from long-term primary and secondary cultures were shown to be responsive to bovine or primate conditioned medium, containing interleukin 2 (IL2), in a dose-dependent manner. Bovine secondary cytotoxic lymphocytes (SCL) were placed in IL2-containing medium in an attempt to generate an IL2-dependent bovine T lymphocyte cell line. SCL retained their alloantigen lytic specificity after two weeks in culture, and are presently still being maintained in IL2-containing medium. Monoclonal antibody B29B, a 'pan' anti-bovine T cell antibody, was shown cause approximately 45% lysis of radiolabelled-bovine SCL in complement-mediated antibody-dependent cytotoxicity assays. The in vitro generation of bovine cytotoxic T lymphocytes has applications in the future examination of the mechanism of bovine CTL-mediated lympholysis. IL2-dependent bovine SCL may be utilized for the generation of antisera specific for cell surface antigens found on bovine CTL, or all bovine T lymphocytes in general, thereby allowing for definitive identification of bovine T cells. Additionally, this in vitro allogeneic mixed leukocyte culture system can be used to study the role of the bovine major histocompatibility complex in allograft responses and may subsequently prove to be useful for the typing of tissues for organ transplantation.

CHAPTER 1

INTRODUCTION

Cytotoxic T lymphocytes have been found to play a central role in the rejection of allografts, tumor immunity, resistance to certain viral infections, and graft vs. host reactions. They have also been suggested to be of major importance in resistance to infection by a variety of organisms, including certain bacteria, fungi, and protozoan parasites. T lymphocytes are one of the two major cellular elements involved in cell-mediated immunity (CMI): the other major component consists of the phagocytic cells, including circulating monocytes, tissue macrophages, promonocytes, and their precursor cells found in bone marrow.

Mononuclear phagocytic cells are involved in the activation of specific T lymphocytes via the modification or specific presentation of antigen to them, and by the production of monokines. Additionally, they are involved in the host's resistance to infection by certain intracellular microorganisms, and in the removal of cell debris and damaged or dying cells (1).

The T cell-mediated immune response is initiated by antigen recognition and subsequent activation of clonally reactive T lymphocytes. Once activated by antigen, T cells serve numerous functions. For example, T-helper cells are required for B lymphocytes to produce antibody. Amplifier T cells produce hormone-like molecules, lymphokines, which augment numerous aspects of the immune response. Killer T cells are capable of specifically recognizing and killing virus-infected cells and tumor cells. And finally, suppressor T cells quench immune reactions.

As T cells mature, differentiate, and become functionally competent, specific molecules are expressed on their cytoplasmic membranes. These cell surface markers are characteristic of specific T cell subsets and have been defined by antisera made against them. In mice, Lyt-antigen expression is confined to T lymphocytes. By utilizing antisera produced in congenic strains of mice, genetically identical except for the loci which coded for the Lyt antigens, Cantor and Boyse (2) discovered that subclasses of peripheral T cells, defined by their functions, express different Lyt surface antigens even before being exposed to immunogens. This demonstrated that T lymphocyte differentiation into distinct subpopulations was a process independent of antigen. In subsequent studies, Cantor and associates (3, 4, 5) determined that T cells expressing Lyt-1 antigens (Lyt-1^{+},2,3^{-} T cells) were responsible for helper/inducer activity in the generation of cytotoxic T cells and in primary antibody responses.

The Lyt-1^-,2,3^+ subclass of T lymphocytes was found to include those which mediated cytotoxic and suppressor activities.

Human T lymphocyte subsets also express distinct cell surface antigens. However, since congenic strains of inbred humans do not exist, the generation of antisera specific for these antigens was not as easy. By immunizing mice with human peripheral T cells or thymocytes, and utilizing a cell fusion technique (6), Reinherz and associates (7, 8, 9) defined subpopulations of human T lymphocytes by monoclonal antibody. They determined that the subclass of human T cells bearing the OKT4 antigen were the helper and amplifier T cells, analogous to the Lyt-1^+,2,3^- T cell subset in mice. The OKT5$^+$ subset was shown to contain T cells with cytotoxic and suppressor functions, resembling the Lyt-1^-,2,3^+ murine T lymphocyte subpopulation.

Studies examining cell-mediated immune responses in cattle have been hampered by a paucity of suitable monospecific reagents to differentiate between the cellular elements involved in such reactions. All investigators seem to agree that bovine B lymphocytes are those cells which have membrane-bound immunoglobulin on their cell surface (sIg$^+$ lymphocytes). However, the identification of bovine T cells has not been as simple. Many groups (10, 11, 12, 13, 14) have identified bovine T lymphocytes as the subpopulation which is sIg^- and which form E-rosettes with neuraminidase-treated (E_N-rosettes) sheep red blood cells (SRBC) and/or 2-aminoethylisothiourium bromide-treated SRBC (E_{AET}-rosettes). Other investigators (14, 15, 16, 17, 18, 19) have defined bovine T cells as those isolated populations of lymphocytes which respond to lectins, such as phytohemagglutinin (PHA) and concanavalin A (ConA), known to be mitogenic for murine and human T lymphocytes.

Pearson, et al (18) studied the effects of various lectins on bovine peripheral blood lymphocytes. In double-labelling experiments using goat anti-bovine immunoglobulin and fluorescein-labelled lectins, the authors determined that the majority of the sIg^- lymphocytes stimulated by "T cell lectins" were also bound by peanut agglutinin (PNA^+ cells), and some bound soybean agglutinin (SBA^+ cells). They suggested utilizing PNA as a cell surface marker for the identification of bovine T lymphocytes. Other investigators (20, 21, 22) have also used PNA-binding as a bovine T lymphocyte marker. Another group (23) has suggested that a blood group A reactive hemagglutinin from the snail Helix pomatia (HP) may be used as a bovine T cell marker after treatment of the lymphocytes with neuraminidase. These authors determined that lymphocytes labelled with HP were sIg^- and formed E_N-rosettes with SRBC.

Of all the methods currently in use for the identification of bovine T lymphocytes, only the PNA or HP labelling techniques give an investigator the ability to distinguish individual cells. Recently, Pinder et al. (24) have generated monoclonal antibodies (McAb) against bovine PBL in an attempt to produce reagents specific for bovine lymphocyte subpopulations. Results from double-labelling experiments, utilizing PNA and McAb, demonstrated that several of the McAb recognized a determinant present on both PNA^+ and PNA^- lymphocytes. One of the McAb was determined to be specific for IgM, and several cross-reacted with lymphocytes from other bovid species. A subsequent study (25) found that two of these McAb reacted with BoLA w6, a bovine major histocompatibility complex (MHC) determinant, but they also cross-reacted with a number of other BoLA antigens. A third McAb showed no BoLA specificity. Much work still needs to be done in this area before bovine T lymphocytes and/or their subpopulations can be defined and identified with consistency and specificity.

One of the in vitro tests used to measure cell-mediated immunity in cattle has been the lymphocyte blastogenesis test (LBT), also called the lymphocyte stimulation test (LST). This assay measures the response of bovine lymphocytes to mitogens or specific antigen. Mitogen stimulation has typically been utilized in the LBT when investigators are examining the effect of some factor, such as age (26), feed (27), or stress (28) on the immune response of the animal. The response of bovine lymphocytes to specific antigen has usually been studied in relation to infection of cattle by a specific agent, such as infectious bovine rhinotracheitis virus (15, 29), Anaplasma marginale (30), Brucella abortus (31), or Theileria parva (32).

There are problems associated with measuring cell-mediated immunity with LBT. There are almost as many different procedures as there are investigators utilizing this assay. Some of the parameters that differ from laboratory to laboratory include: whole blood cultures vs. isolated peripheral blood leukocytes (PBL); incubation time and temperature for both the length of the assay and the duration of radionucleotide labelling; the nature and amount of radioisotope; cell culture media; the addition and concentrations of various media additives such as 2-mercaptoethanol or serum; and the method of reporting results. All of these diverse factors make it difficult to compare the results obtained by different investigators. Additionally, as Schultz (33) has suggested, during LBT the cell populations being examined are probably not T effector cells in CMI, but rather T helper cells and/or T suppressor cells.

Another in vitro assay used to measure CMI in cattle has been the leukocyte migration-inhibition test (33, 34), but, again, the mechanics of the assay itself and the methods of measurement vary a great deal from one report to another, making it difficult to compare results obtained by different laboratories.

In theory, the lymphocyte-mediated cytotoxicity (LMC) assay most closely correlates with T cell-mediated immunity in vivo (35). The mechanisms involved in T cell-mediated lympholysis have been extensively studied in the murine system (36). However, very little is known about these responses in cattle. Recently, Pearson, et al. (37) reported the generation of bovine cytotoxic lymphocytes from animals immune to the protozoan parasite Theileria parva (T. parva) after in vitro culture with X-irradiated T. parva-infected autologous lymphocytes. Cytotoxic activity was neither observed when PBL taken directly from the immune animals were used as effector cells, nor when PBL from normal (non-infected, non-immune) animals were stimulated with autologous T. parva-transformed cells in MLC, then used as effector cells. Thus, the cytotoxic cells generated in this study were essentially from a secondary in vitro stimulation of PBL initially primed in vivo with T. parva.

Subsequent studies by Eugie and Emery (38) and Emery and associates (39) also determined that cytolytic cells are found in cattle immune to T. parva. The cytotoxicity observed in these studies was apparently genetically restricted to infected autologous cells. In the former study, Eugie and Emery (38) found that immune calves rechallenged with either T. parva sporozoites or autologous T. parva-infected cells exhibited cytotoxic responses in PBL that were restricted to infected autologous target cells. During a primary T. parva infection, Emery et al. (39) determined that spontaneous nonspecific cytotoxic activity of PBL occurred ten days after challenge, lysing both allogeneic infected cell lines and a murine lymphoma cell line. However, this nonspecific lysis was only observed during lethal infection of calves. PBL from immune calves were specifically cytotoxic for autologous cell lines infected by T. parva, exhibiting less than five percent lysis of allogeneic or xenogeneic target cells. The authors speculated that the nonspecific lysis they observed during the primary infection of animals may have been due to natural killer (NK) cell-mediated cytotoxicity. In addition, it appeared that at least two weeks were required after the initial immunization before the cell-mediated immune response against T. parva was able to afford the host specific protection against rechallenge with the organism.

Emery and associates (20) attempted to characterize the effector cell mediating cytotoxicity in this system by examining the effects of removal of various populations of bovine PBL on the cytotoxic response to T. parva-infected cells. Depletion of adherent cells, adherent cells and

Fc/C3 receptor bearing cells, or sIg^+ cells and adherent cells did not produce a significant decrease in the cytotoxic response against T. parva infected cells, and, in some cases, slightly enhanced cytotoxicity. Recovered adherent cells or Fc/C3 receptor-bearing cells mediated very little lysis when examined alone. Since maximum cytotoxicity was mediated by PNA^+ or SBA^+ lymphocytes, the authors concluded that the cells effecting lysis of T. parva-infected cells were bovine T lymphocytes.

Pearson, et al. (40) found that, although both autologous and allogeneic T. parva-infected cell lines induced a strong proliferative response in PBL from both normal and immune cattle during mixed leukocyte reactions, they were only able to induce a cytotoxic response when the responding population was from immune animals. Studying different responder/stimulator cell MLC combinations, and utilizing cold-target inhibition techniques, the authors determined that both genetically restricted and non-restricted components were present, and this was likely due to distinct effector cell populations.

In sharp contrast to the above studies, Emery and Kar (41) were able to generate cytotoxic cells during the in vitro culture of normal PBL responder cells with autologous T. parva-transformed stimulator cells. Examination of the specificity of these cytotoxic cells by blocking studies revealed that both genetically-restricted (CTL) and natural killer-like activities had been generated. However, CTL obtained from immune cattle only exhibited genetically-restricted lysis of autologous T. parva-infected cells.

Upon examination of the literature concerning bovine T lymphocytes, a number of questions arise. Why should bovine T cells form rosettes with sheep erythrocytes? Both murine and human T lymphocytes have been shown to be responsive to certain lectins such as PHA and ConA, yet does this fact necessarily hold true for bovine T cells? Are PNA^{+} bovine lymphocytes exclusively T cells just because no surface immunoglobulin is expressed on their cell membranes? If this is true, does PNA label all T lymphocytes, or certain subpopulations? One of the major criteria for the identification of T cells in other systems has been the function(s) performed by these cells. For example, cytotoxic T lymphocytes are defined as those lymphocytes which mediate antigen-specific lysis of target cells. This differentiates CTL from cells involved in nonspecific cellular cytotoxicity, such as natural killer cells. The same definitive identification should be utilized in research involving bovine T lymphocytes.

The present study examined the allogeneic mixed leukocyte culture system for the in vitro generation of alloantigen-specific bovine cytotoxic T lymphocytes. Highly specific lytic activity was consistently produced after secondary restimulation of long-term primary cultures. Bovine secondary cytotoxic lymphocytes (SCL) were subsequently placed into medium containing a known source of IL2 and are presently being maintained in IL2-dependent culture. These bovine SCL have the potential to be utilized in generating antisera specific for cell surface antigens present exclusively on bovine cytotoxic T lymphocytes, antigens found on all bovine T lymphocytes, i.e. "Thy-1-like" antigens, and/or bovine MHC antigens. In addition, this in vitro system could further be used to examine the mechanism(s) of bovine T cell-mediated lympholysis and the role of CTL in a variety of immune responses of cattle, such as the host's resistance to intracellular infection and tumor immunosurveillance.

CHAPTER 2

MATERIALS AND METHODS

Medium

Powdered RPMI 1640 medium (Cat. no. 430-1800, Gibco, Grand Island, NY) was prepared in distilled, deionized water with inclusion of $NaHCO_3$, 24 mM, and N-2-hydroxyethylpiperizine-N'-2-ethanesulfonic acid (HEPES), 25 mM, (Cat. no. 16926, U.S. Biochemicals, Cleveland, OH), filtered through a 0.22 um filter, the osmolarity adjusted to 300 mOsm, and stored at $4^{o}C$ until used. Prior to use, it was supplemented with 2-mercaptoethanol (Cat. no. M6250, Sigma, St. Louis, MO), 50 uM; glutamine (Cat. no. 320-5030, Gibco), 2 mM; penicillin-G (Pfizerpen, Cat. no. 1622, Pfizer Inc., New York, NY), 50 IU/ml; gentamicin (Garamycin, NDC no. 0085-0069-03, Schering Pharmaceutical Corp., Kenilworth, NJ), 50 ug/ml; l-alanine (Cat. no. A7627, Sigma), 20 ug/ml; l-asparagine (Cat. no. A0884, Sigma), 17.4 ug/ml; l-aspartic acid (Cat. no. A9256, Sigma), 24 ug/ml; l-glutamic acid (Cat. no. G1251, Sigma), 60 ug/ml; l-proline (Cat. no. P0380, Sigma), 32 ug/ml; sodium pyruvate (Cat. no. 890-1840, Gibco), 88 ug/ml; biotin (Cat. no. B4501, Sigma), 0.136 ug/ml; Vitamin B_{12} (Cat. no. V2876, Sigma), 0.136 ug/ml; and fetal bovine serum (FBS, Sterile Systems, Logan UT), 10% (v/v). This medium will hereafter be referred to as supplemented RPMI 1640 medium.

A modification of Iscove's totally defined, serum-free medium (IMDM) was prepared as previously described (42). Briefly, powdered Dulbecco's modified Eagle's medium (DMEM, Cat. no. 430-2100, Gibco) was dissolved to approximately 1.25x concentration in distilled, deionized water. It was then supplemented with the following: $NaHCO_3$, 31 mM; HEPES (U.S. Biochemicals), 25 mM; l-cystine-HCl (Cat. no. C8755, Sigma), 120uM; fatty acid-free BSA (Cat. no. A7511, Sigma), 14.5 uM; Na_2SeO_3 (Cat. no. S1382, Sigma), 160 uM; 2-mercaptoethanol (Sigma), 50 uM; human transferrin (Cat. no. 616397, Calbiochem-Behring, San Diego, CA), 1.13 mM, 1/3-saturated with $FeCl_3$; l-alanine (Sigma), 222 uM; l-asparagine (Sigma), 131 uM; l-aspartic acid (Sigma), 180 uM; l-glutamic acid (Sigma), 410 uM; l-proline (Sigma), 8.7 mM; sodium pyruvate (Gibco), 8.7 mM; biotin (Sigma), 23 uM; Vitamin B_{12} (Sigma), 4.0 uM; l-glutamine (Gibco), 2.0 mM; penicillin (Pfizer), 50 IU/ml; and gentamicin (Schering), 50 ug/ml. A suspension of cholesterol (Cat. no. CH-S, Sigma), 19 uM, linoleic acid (Cat. no. L1376, Sigma), 10 uM, and 1-oleoyl-2-palmitoyl phosphatidylcholine (Cat. no. P4142, Sigma), 1.0 mM was prepared in 1X DMEM containing 290 uM fatty acid-free BSA (Sigma). This suspension was sonicated at 4°C for 10-12 min at 80 watts and added to the previous mixture at a ratio of 1:400 (v/v). This medium was brought to 1X concentration with distilled, deionized water, the pH adjusted to 7.1, and the osmolarity adjusted to 300 mOsm. It was then filtered through a 0.22 um filter and stored in 500 ml aliquots at -20°C until used.

KC-100 medium, an experimental serum-free medium, was generously provided by KC Biologicals, Lenexa, KS.

Cells

Bovine peripheral blood leukocytes (PBL) were from cattle maintained at the Veterinary Research Laboratory, Montana State University, Bozeman, MT. Animal no. 1 was a three-year-old Angus heifer and no. 32 was a three-year-old Hereford steer. Animal no. 60 was a two-year-old Hereford steer and no. 925 was a seven-year-old Angus cow. The PBL from these cattle were harvested as previously described (42) with some modification. Briefly, the buffy coat from 50-500 ml of heparinized (10 IU/ml) venous blood was removed and diluted to two to four volumes in phosphate buffered saline (PBS) without calcium or magnesium (Cat. no. 310-4200, Gibco). Ten ml aliquots of this cell suspension were layered onto 4 ml of Ficoll-Hypaque (Histopaque, Cat no. 1077-1, Sigma) and centrifuged for 35-45 min at 350 xg. The PBL-rich band was removed, washed twice in PBS, counted via a Coulter electronic cell counter, and adjusted to the desired concentration in medium as indicated. When PBL clumping occurred between washings, PBS containing heparin (5 IU/ml) was used after disruption of clumps by passage through a 20 gauge needle.

MLA, a retrovirus-infected primate lymphocyte cell line, was generously provided by Dr. Gary Splitter, University of Wisconsin-Madison, Madison, WI. MLA cells were grown in RPMI 1640 medium and found to constituitively produce a factor, primate interleukin 2 (IL2), capable of maintaining alloantigen-primed bovine lymphocytic cells in culture (Dr. Gary Splitter, personal communication).

Conditioned Medium (CM)

Conditioned media were prepared as described previously (22). Briefly, bovine PBL were adjusted to 10^7 cells/ml in IMDM and cultured with 5.0 ug/ml ConA (Miles Biochemicals) at 37°C for 24-48 hr in a humidified atmosphere of 5% CO_2 in air. At that time, cells were removed by centrifugation, and CM was filtered through a 0.22 um filter and stored at 4°C until used.

Primary Mixed Leukocyte Reaction (MLR)

Bovine PBL were harvested as described above and adjusted to 2.5×10^6 cells/ml in KC-100 medium with 25 ug/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) and cultured for

68-72 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. These "stimulator" cells were harvested by centrifugation for 10 min at 350xg. In order to inhibit subsequent cellular replication, the cell pellet was resuspended in 10 ml of RPMI 1640 medium and mitomycin-C (Cat. no. M0503, Sigma) was added to a final concentration of 30 ug/ml. The cells were incubated at 37°C for 1 hr, then centrifuged at 350xg and the pellet was resuspended in 1-2 ml RPMI 1640 medium. This suspension was carefully layered over 10 ml FBS and centrifuged at 350xg for 10 min. The cell pellet was resuspended in 1-2 ml of RPMI 1640 medium and the FBS wash was repeated. The cells were then resuspended, counted, and adjusted to 2×10^6 cells/ml in supplemented RPMI 1640 medium.

"Responder" cells were fresh PBL, prepared as described above, and adjusted to 2×10^6 cells/ml in supplemented RPMI 1640 medium.

Triplicate samples of responder and stimulator cells were added in 100 ul aliquots to 96-well flat-bottomed microtiter plates (Linbro, Cat. no. 76-032-05, Flow Laboratories, Inc., McLean, VA), and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. At times indicated, 50 ul of autologous culture medium containing 10 uCi/ml of methyl-tritiated thymidine ([^{3}H]-Tdr, Cat. no. NET-027A, New England Nuclear, Boston, MA) were added to each well. After an additional 4 hr incubation at 37^{o}C, contents of the individual wells were harvested onto glass fiber filter strips using a PHD Cell Harvester (Cambridge Technology, Inc., Cambridge, MA). After the filters had air dried, a toluene-based scintillation cocktail (Liquifluor, Cat. no. NEF-903, New England Nuclear) was added and radionucleotide incorporation was quantified using a Beckman LS 100C liquid scintillation counter or a Packard Tri-Carb 460 liquid scintillation counter serially connected to an IMS 8000 SX microcomputer. Data reduction was accomplished using a BASIC program designed to compute the means and standard deviations of the samples (43).

Primary Mixed Leukocyte Culture (MLC):

Ten ml each of the responder and stimulator cell populations were cultured upright in cell culture flasks (Cat. no. 3012, Falcon). Alternatively, 1 ml aliquots of each cell population were cultured in 24-well cluster plates (Cat. no. 3524, Costar). In either case, cultures were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 in air for times indicated.

Secondary Mixed Leukocyte Reaction

Responder cells were harvested from primary MLC at times indicated, and viable cells were recovered by centrifugation of the resuspended cultures over Ficoll-Hypaque as previously described. The cells were washed twice by centrifugation in RPMI 1640 medium or PBS, counted via a Coulter electronic cell counter, and adjusted to $2x10^6$ cells/ml in supplemented RPMI 1640 medium.

Stimulator cells were mitomycin-C-inactivated LPS blasts, prepared as described above, and adjusted to $2x10^6$ cells/ml in supplemented RPMI 1640 medium.

Triplicate samples of responder and stimulator cells were added in 100 ul aliquots to flat-bottomed microtiter plates (Linbro), and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. At times indicated, 50 ul of autologous culture medium containing 10 uCi/ml [^{3}H]-Tdr were added to each well. After 4 hr incubation, the contents of the wells were harvested, and radionucleotide incorporation was quantified as described for the "Primary Mixed Leukocyte Reaction."

Secondary Mixed Leukocyte Culture

One ml aliquots of the responder and stimulator cell populations were added to 24-well cluster plates (Costar) and incubated at 37°C 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 and Smith (44), with minor modifications. Effector cells were harvested from primary or secondary MLC at 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 via a Coulter electronic cell counter. Log_2 dilutions were made, and 100 ul aliquots of each dilution were added to triplicate wells of conical (Cat. no. 76-023-05, Linbro) or flat-bottomed (Linbro) microtiter plates.

Target cells were prepared by culturing fresh bovine PBL in 75 cm^2 flasks (Corning) at 10^6 cells/ml in IMDM medium with 0.31 ug/ml ConA (Miles Biochemicals) for 48 hr (42). Viable ConA blasts were harvested by centrifugation over Ficoll-Hypaque, washed twice in RPMI 1640 medium or PBS, and resuspended in 5-7 drops of FBS. After addition of 350 uCi of ^{51}chromium (^{51}Cr, as sodium chromate, $Na_2{}^{51}CrO_4$, Cat. no. NEZ-030S, New England Nuclear), target cells were incubated at 37°C for 1-2 hr. Excess ^{51}Cr was removed by pelleting the cells, resuspending them in approximately 1-2 ml RPMI 1640 medium, layering the suspension over 10 ml FBS, centrifuging for 10 min at 350xg, then repeating the FBS wash. Cells were resuspended in supplemented RPMI 1640 medium, counted, and adjusted to give an effector:target cell ratio of at least 100:1. Aliquots of 100 ul were added to the microtiter plates already containing the effector cells. The plates were centrifuged at 200xg for 10 min at room temperature and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 4 hours.

The ^{51}Cr release reaction was stopped by a 350xg centrifugation at 2°C for 10 min. A 100 ul supernatant sample from each well of the triplicate cultures was added to 3.5 ml Biofluor Scintillant (Cat. no. NEF-961, New England Nuclear) and counted on a Beckman LS 100C liquid scintillation counter or a Packard Tri-Carb 460 liquid scintillation counter serially connected to an IMS 8000 SX microcomputer. Data reduction was accomplished using a BASIC program to compute the means and standard deviations of stored data (43).

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 (six drops of Lyzerglobin [Cat. no. JD12268-1, VWR] added to 10 ml ISOTON II diluent [Cat. no. 357-212, Curtin Matheson Scientific, Inc., Hanover, PA]). Percent specific lysis was determined using the following formula:

Percent specific lysis =

$$\frac{\text{mean sample cpm} - \text{mean spontaneous cpm}}{\text{mean maximum cpm} - \text{mean spontaneous cpm}} \times 100\%$$

where cpm represents counts per minute.

Complement-Mediated Antibody-Dependent Cytotoxicity (CMADC) Assay

Effector cells were labelled with ^{51}Cr according to the method described above, and resuspended in supplemented RPMI 1640 medium containing 10% (v/v) heat-inactivated (56°C for 30 min) FBS (HI-FBS, Sterile Systems). These cells were then counted and adjusted to at least $2x10^5$ cells/ml. The monoclonal antibody B29B, produced in ascites, was a kind gift from Dr. William Davis, Washington State University, Pullman, WA. B29B had previously been demonstrated to be a pan anti-bovine T cell antibody (William Davis, submitted for publication). Rabbit complement (C', Cat. no. 31042) was purchased from Pel-Freez Biologicals, Rogers, AR.

Aliquots of 100 ul of ^{51}Cr-labelled effector cells were added to conical microtiter plates (Linbro). Dilutions of B29B antibody were made as indicated in RESULTS, and 50 ul aliquots of each dilution or 50 ul supplemented RPMI 1640 medium plus HI-FBS were added to triplicate wells of the microtiter plates. After incubation in a humidified atmosphere of 5% CO_2 in air at 37°C for 30 min, 50 ul aliquots of supplemented RPMI 1640 medium plus HI-FBS or a dilution(s) of active rabbit C' were added to appropriate wells. The plates were incubated at 37°C for an additional 90 min, then centrifuged at 350xg at 2°C for 10 min to halt the ^{51}Cr release reaction. A 100 ul supernatant sample from each well of the triplicate cultures was added to 3.5 ml Biofluor Scintillant (New England Nuclear) and counted on a Beckman LS 100C or a Packard Tri-Carb 460 liquid scintillation counter, as before.

Maximum and spontaneous release from the ^{51}Cr-labelled effector cells was determined as previously described for the LMC assay. Percent specific lysis was also calculated as indicated earlier.

Alternatively, unlabelled effector cells were resuspended in supplemented RPMI 1640 medium plus HI-FBS, the cell concentration was determined via a Coulter electronic cell counter, and viability determined by microscopic observation of trypan blue exclusion. Aliquots of 50 ul were added to conical microtiter plates (Linbro). Additions of B29B antibody, active rabbit C', and medium were the same as described above.

Aliquots of 50 ul of ^{51}Cr-labelled target cells, prepared as described previously, were added to the microtiter plates. The remainder of the CMADC assay was performed as described for the LMC assay.

<u>Response to Exogenous Interleukin 2 (IL2)</u>

Viable effector cells from primary or secondary MLC were harvested by centrifugation over Ficoll-Hypaque as before, and 100 ul aliquots were added to 96-well flat-bottomed microtiter plates (Linbro). Samples (100 ul) of MLA supernatant fluids or CM were added to a final concentration of 20% (v/v) in triplicate wells. Alternatively, serial $\log_2$ dilutions of 100 ul samples of MLA supernatant fluids or CM were made in 11 individual wells of flat-bottomed microtiter plates (Linbro). The last well of each row served as a medium control. Then 100 ul aliquots of effector cells were added to each well, and the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

At times indicated, 50 ul of autologous culture medium containing 10 uCi/ml [^{3}H]-Tdr (New England Nuclear) were added to each well and the plates were incubated for an additional 4 hr. Contents of individual wells were harvested and radionucleotide incorporation determined as described for the "Primary Mixed Leukocyte Reaction."

CHAPTER 3

RESULTS

Primary Mixed Leukocyte Reaction

The primary *in vitro* proliferative response of bovine peripheral blood leukocytes (PBL) to alloantigen was examined. Results from a representative experiment are shown in Table 1. Peak proliferation was found to occur at approximately day 5-6 of the mixed leukocyte reaction (MLR). The response was specific for the alloantigen as indicated by the low amount of [^{3}H]-Tdr incorporated by the responder population when stimulated with autologous mitomycin-C-inactivated LPS blast cells.

Cytotoxic Response in Primary Culture

Viable effector cells from primary MLC were examined for cytotoxic activity on various days in three separate experiments. Three target cell populations were tested in the lymphocyte-mediated cytotoxicity (LMC) assays: 1.) Allogeneic concanavalin A (ConA)-induced blast cells from the same animal as the original stimulating cells; 2.) Autologous ConA blast cells from the responding animal; and, 3.) Third party ConA blast cells from an animal not utilized in the original primary MLC.

Table 1. Primary Mixed Leukocyte Reaction: Responder cells (1) were cultured with allogeneic (32^m) or autologous (1^m) mitomycin-C-inactivated stimulator cells.

	Time After Stimulation		
	Day 4	Day 5	Day 6
$1 + 32^m$	19,714 ± 5,135[a]	80,576 ± 8,257	80,859 ± 14,356
$1 + 1^m$	3,473 ± 577	4,808 ± 208	2,349 ± 1,266
32^m + Medium	894 ± 233	2,001 ± 1,441	1,206 ± 493
1^m + Medium	864 ± 197	1,665 ± 554	700 ± 324

[a] Results are expressed as the mean cpm $[^3H]$-Tdr incorporation of triplicate cultures ± S.D.

The maximum cytolysis observed in all experiments occurred on day 5 (Table 2). However, significant lysis of allogeneic target cells, seen in experiment 2, could not consistently be reproduced.

Table 2. Maximum percent cytolysis (% Cyto) of target cells observed at the highest effector to target cell ratio (E:T) on day 5 of primary MLC.

	Effector Cells vs.					
	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	-6	101	>1

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. In order to minimize variability, a series of MLC flasks were simultaneously established, serving as a source of effector cells for subsequent LMC assays. As before, three target cell populations were included in each assay: 1.) Allogeneic ConA blast cells; 2.) Autologous ConA blast cells; and 3.) Third party ConA blast cells.

On day 5 (Figure 1) of the MLC, approximately 19% specific lysis of the allogeneic target cells was seen at the highest effector:target cell ratio examined (161:1). Nearly 9% and 10% lysis was seen at the highest effector:target cell ratios for the autologous and third party target cells, respectively. Specific lysis of the allogeneic target cells had increased to 37.5% by day 10 of the primary MLC, whereas there was less than 2% lysis of the autologous target cells and approximately 7% lysis of the third party target cells at the highest effector:target cell ratios tested (165:1 and 167:1, respectively) (Figure 2).

By day 15 (Figure 3), the cytotoxic response against the allogeneic target cells in the primary MLC had peaked at approximately 50% lysis and then decreased to about 36% by day 20 (Figure 4). The cytotoxic response of the bovine cells after primary stimulation showed antigen specificity as seen by the low cytolysis of both the autologous and third party target cells as compared to allogeneic target cells (Figures 1-4).

Figure 1. Primary LMC Assay: Effector cells were harvested from primary (1X32) MLC on day 5.

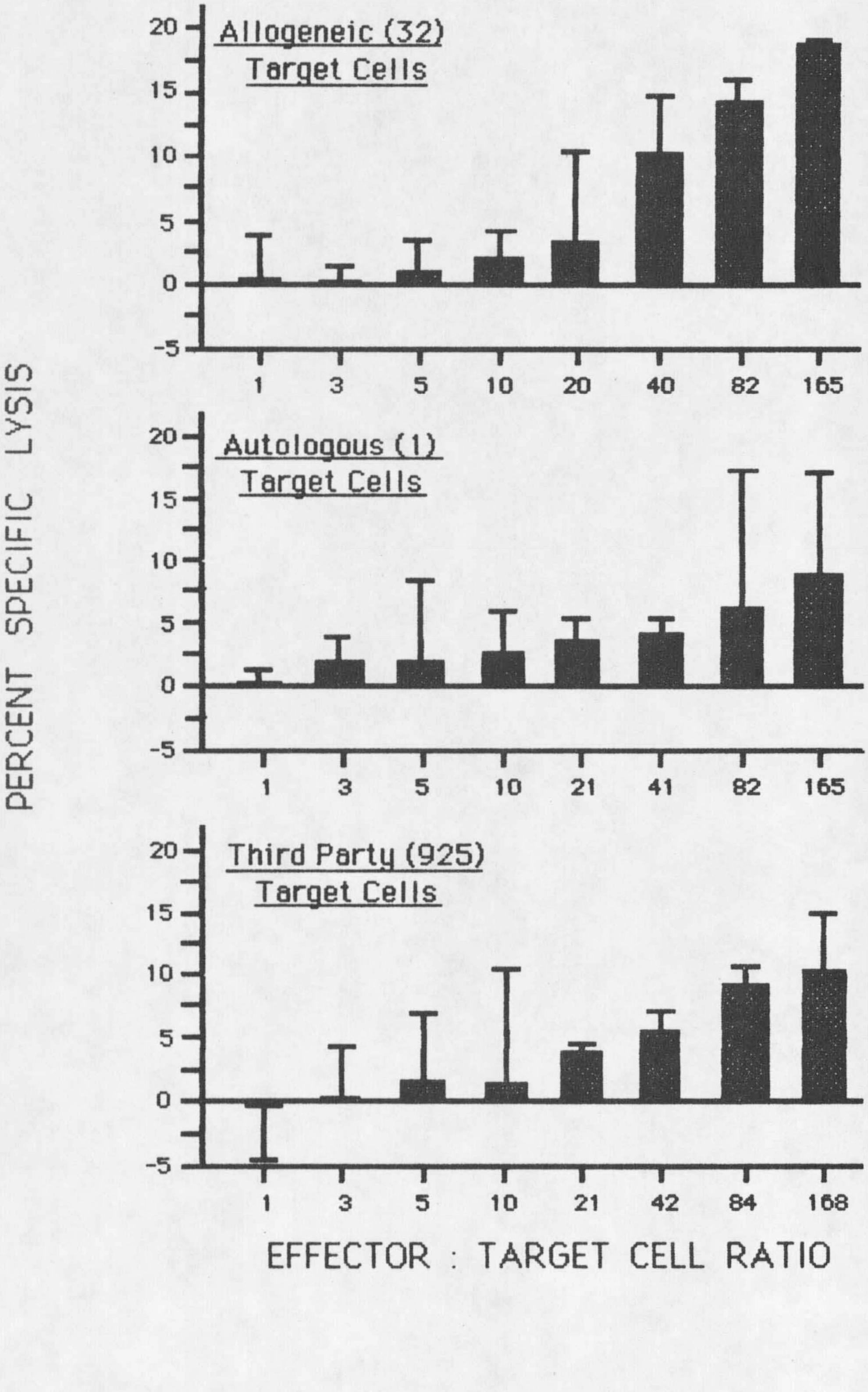
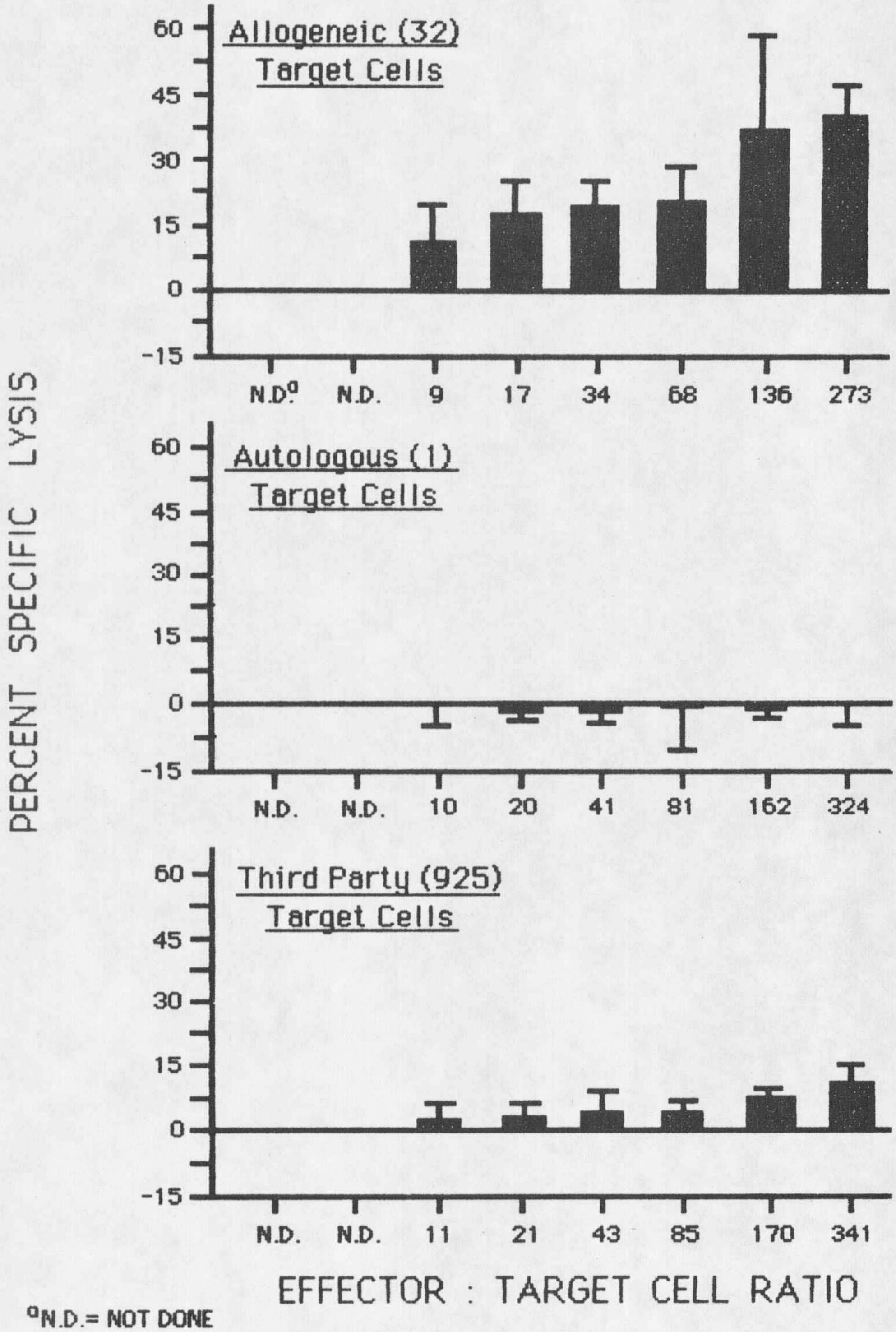


Figure 2. Primary LMC Assay: Effector cells were harvested from primary (1X32) MLC on day 10.



[a]N.D.= NOT DONE

Figure 3. Primary LMC Assay: Effector cells were harvested from primary (1X32) MLC on day 15.

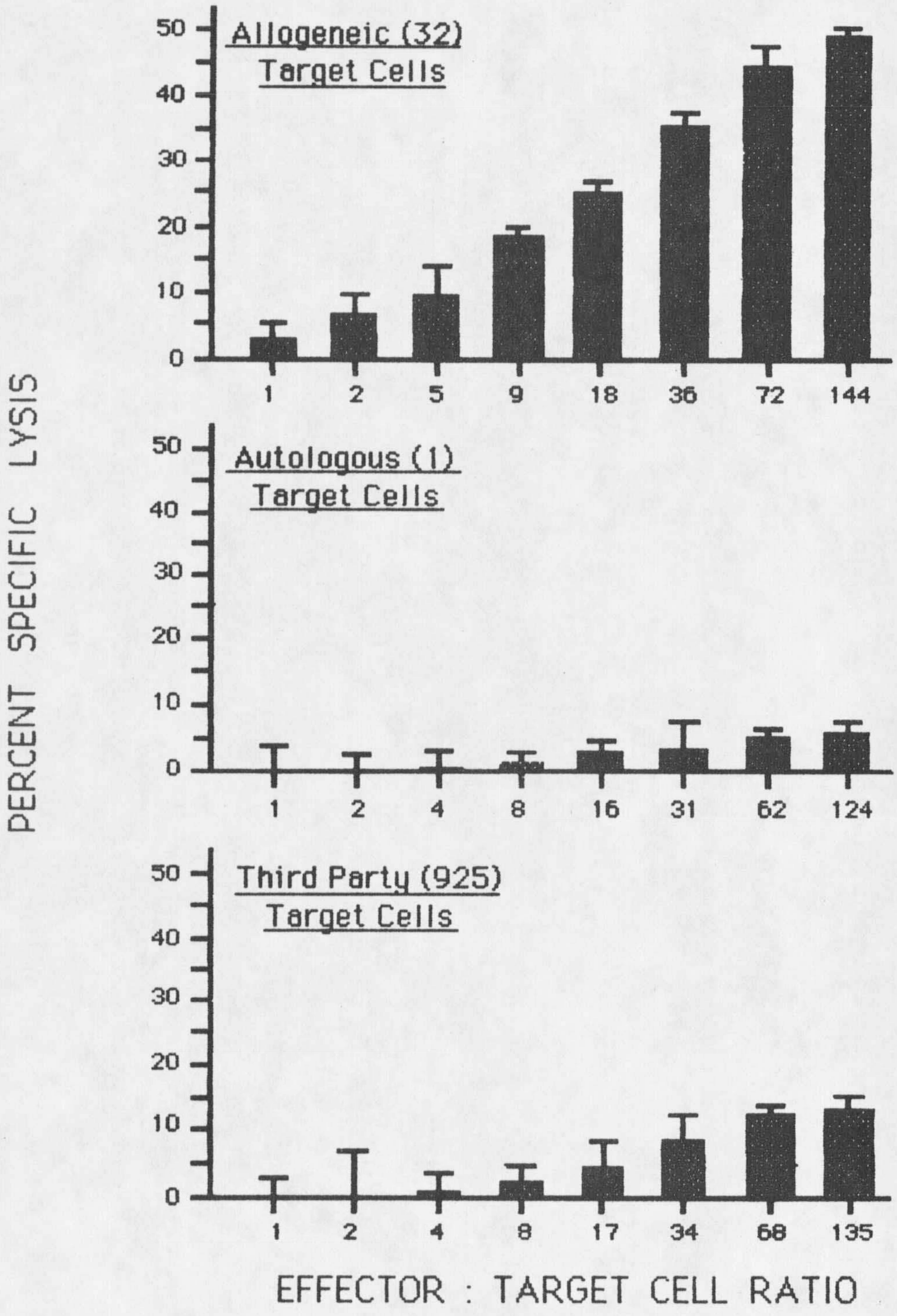
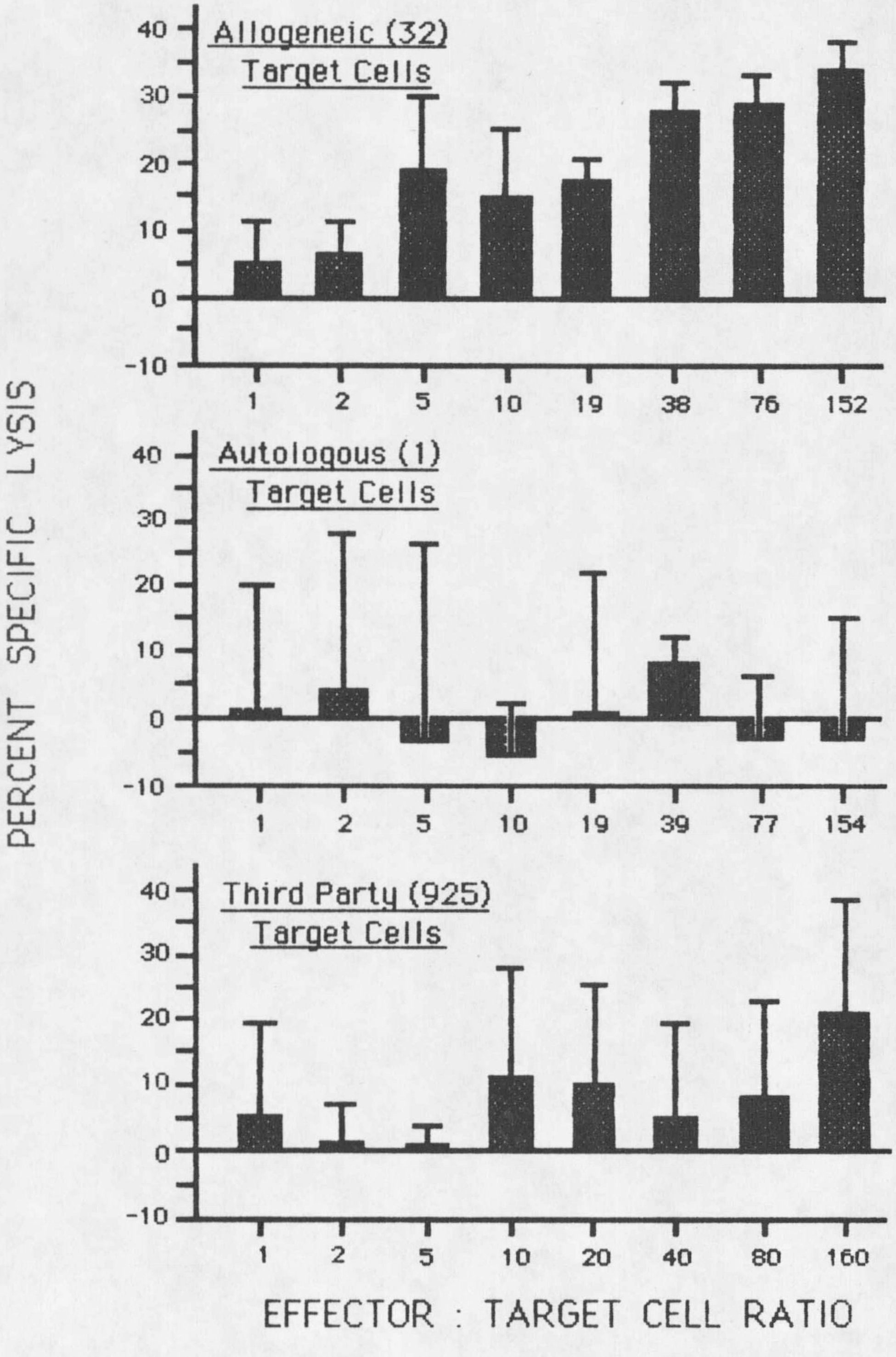


Figure 4. Primary LMC Assay: Effector cells were harvested from primary (1X32) MLC on day 20.



As the cytotoxic response increased over time, the proliferative response of these cells decreased, as shown by the incorporation of [^{3}H]-Tdr during primary mixed leukocyte reaction (Table 3). In addition, the cytotoxic activity showed a linear dose-response relationship with decreasing $\log_2$ dilutions of effector cells.

Response of Cells in Primary Long-Term Culture to Bovine or Primate Interleukin 2

Cells cultured for 20 days were examined for responsiveness to exogenous bovine and primate CM, containing interleukin 2 (IL2). These viable cells incorporated 87,856 ± 1,921 and 43,330 ± 2,275 cpm [^{3}H]-Tdr four days after the addition of MLA supernatant fluids and bovine CM (a source of bovine IL2 [22, 42]), respectively. On the other hand, identical effector cells incorporated only 629 ± 189 cpm [^{3}H]-Tdr when cultured in medium alone (Figure 5). In addition, with each source of bovine or primate CM tested, there was a strict CM dose-dependent incorporation of [^{3}H]-Tdr into the viable cells from primary long-term MLC (Figure 6).

Secondary Mixed Leukocyte Reaction

Viable responder cells from a primary day 15 MLC were examined for proliferation upon restimulation with allogeneic or autologous mitomycin-C-inactivated LPS blasts.

Table 3. Primary Mixed Leukocyte Reaction: Responder cells (1) were cultured with allogeneic (32^m) or autologous (1^m) mitomycin-C-inactivated stimulator cells.

	Time After Stimulation			
	Day 5	Day 6	Day 10	Day 15
$1 + 32^m$	13,122 ± 7,651[a]	25,996 ± 10,693	8,865 ± 3,274	8,519 ± 3,060
$1 + 1^m$	909 ± 316	816 ± 65	3,965 ± 3,502	1,756 ± 1,132
32^m + Medium	159 ± 70	343 ± 242	162 ± 14	236 ± 143
1^m + Medium	250 ± 170	255 ± 118	118 ± 60	233 ± 19

[a] Results are expressed as the mean cpm $[^3H]$-Tdr incorporation of triplicate cultures ± S.D.

Figure 5. Response of viable cells in primary long-term culture to bovine or primate IL2: Cells were cultured in the concentration of bovine (B86) or primate (MLA) CM normally used in cell culture (20% v/v).

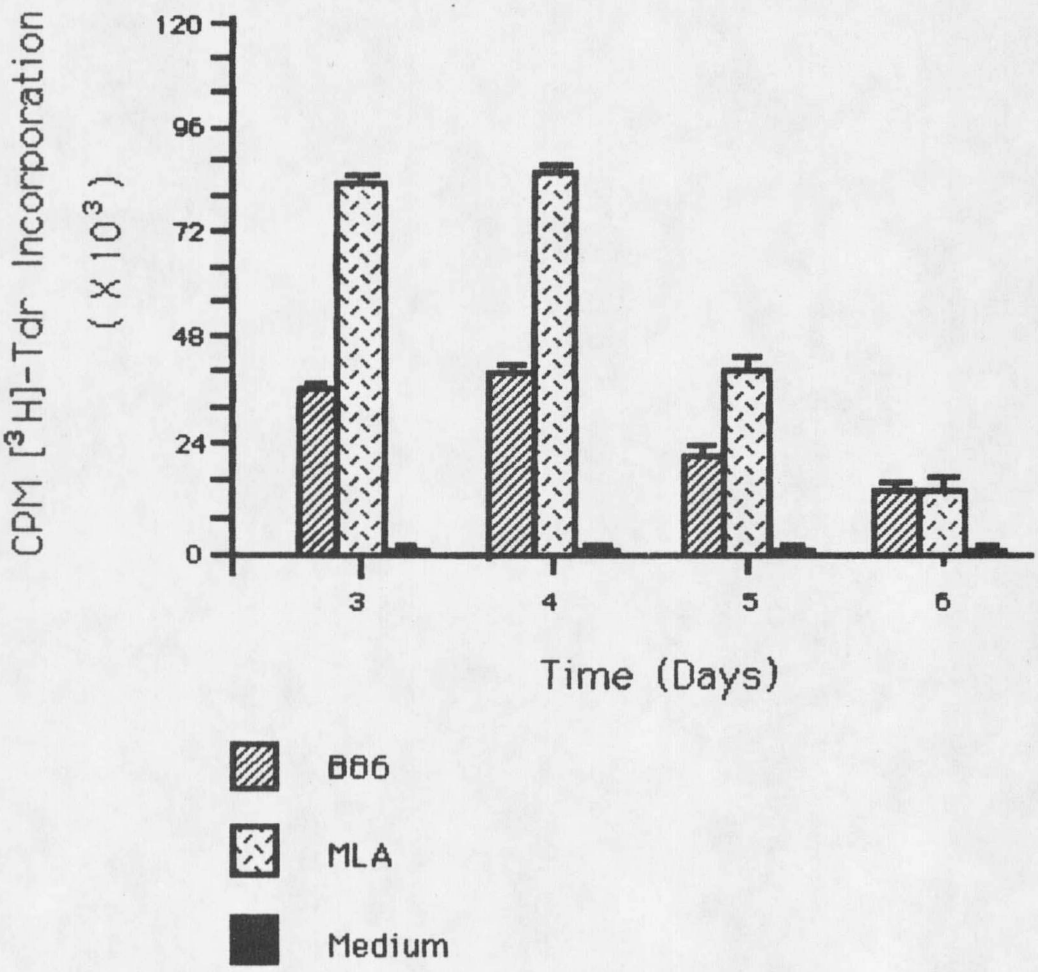
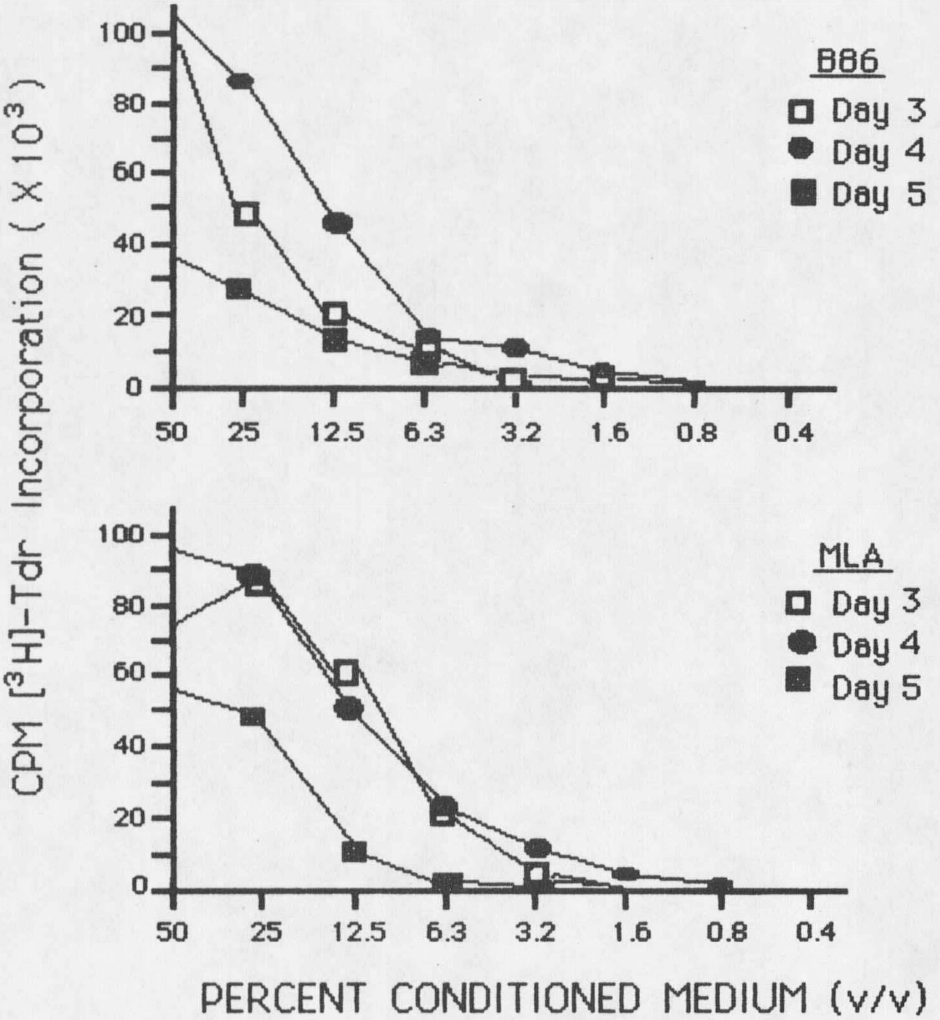


Figure 6. Response of viable cells in primary long-term culture to bovine or primate IL2: Cells were cultured in serial log_2 dilutions of bovine (B86) or primate (MLA) CM.



The peak secondary proliferative response occurred three days later (Table 4). The responding population incorporated 179,216 ± 3,533 cpm of [^{3}H]-Tdr when restimulated with alloantigen, as compared to 30,728 ± 9,075 cpm upon restimulation with autologous LPS blasts, and 1,265 ± 786 cpm when incubated with culture medium alone. The allogeneic stimulator cells incorporated only 180 ± 56 cpm [^{3}H]-Tdr.

The proliferative response was similarly alloantigen-specific when the responding population for the secondary MLR was harvested on the 20th day of primary culture (Table 5).

Cytotoxic Response in Secondary Culture

The lytic activity of the responding population was measured at various points in time after secondary restimulation with alloantigen. Results from representative experiments are shown in Figures 7, 8, and 9. Effector cells harvested three days after the secondary restimulation of a primary day 20 MLC (Figure 7) exhibited approximately 81% specific lysis against the allogeneic target cells. These cells showed a high degree of antigen specificity, lysing 16% and less than 5% of the third party and autologous target cells, respectively. Maximum antigen-specific lysis occurred at the highest effector:target cell

Table 4. Secondary Mixed Leukocyte Reaction: Viable responder cells from day 15 primary MLC (1X32) were restimulated with allogeneic (32^m) or autologous (1^m) mitomycin-C-inactivated LPS blast cells.

	Time After Restimulation		
	Day 3	Day 4	Day 5
(1X32) + 32^m	179,216 ± 3,533[a]	81,185 ± 16,402	19,824 ± 2,959
(1X32) + 1^m	30,278 ± 9,075	29,844 ± 19,216	12,968 ± 6,137
(1X32) + Medium	1,265 ± 786	753 ± 424	1,020 ± 832
32^m + Medium	180 ± 56	231 ± 69	205 ± 19
1^m + Medium	99 ± 53	180 ± 39	265 ± 94

[a] Results are expressed as the mean cpm [^{3}H]-Tdr incorporation of triplicate cultures ± S.D.