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Development and maintenance of bovine cytotoxic lymphocytes with recombinant human interleukin-2

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SUMMARY

Long-term bovine lymphocyte cultures were initiated by stimulation with alloantigens and maintained in continuous culture using medium containing recombinant human interleukin-2 (rh IL-2). The development of specific and lectin-dependent killing wa^{c} monitored following primary alloantigen challenge. Cytolytic activity was barely detectable after 7 days of culture, but gradually increased with peak activity occurring after 21 days of culture. A panel of monoclonal antibodies (MoAb) was used to determine whether a shift in the antigen phenotype of the cell population occurred during culture. The primary cell type that grew in culture was of the T-cell lineage with minimal or no expression of class II antigens. The activities of adenosine deaminase (ADA), purine nucleotide phosphorylase (PNP), adenosine kinase (AK), deoxyadenosine kinase (dAK), deoxycytidine kinase (dCK), 5′-nucleotidase (5′-N), AMP deaminase, hypoxanthine-guanine phosphoribosyl transferase (HGPRT or HPRT), and adenine phosphoribosyl transferase (APRT) were measured by microassay in resting peripheral blood lymphocytes (PBL) and in cells from long-term cultures. Large increases in the activities of PNP and HPRT with a decrease in the activity of ADA were observed. The data show that long-term cultures of lymphocytes can be readily generated, and that sequential changes in antigenic phenotype and function can be monitored and correlated with quantitative changes in enzyme activity.

INTRODUCTION

Cytotoxic T lymphocytes (CTL) may play an important role in disease resistance by directly killing virus-infected and neoplastic cells. Activation of these cells by antigen results in the expression of IL-2 receptors with subsequent proliferation in the presence of IL-2 (Larsson & Coutinho, 1979; Gillis & Smith, 1977). When IL-2 is administered to cell cultures for extended periods of time, IL-2 responsive cells, which include activated T lymphocytes (Gillis & Smith, 1977) and natural killer (NK) cells, preferentially proliferate (Brooks, Urdal & Henney, 1983).

Identification of CTL in other species has been accomplished using MoAb; in addition, attempts are being made to utilize specific enzymes as T-cell differentiation markers. Analysis of enzymes associated with purine salvage exhibit restricted distribution in different lymphocyte subpopulations (Ma *et al.*, 1982; Van Laarhoven, Spierenburg & De Bruyn, 1980). These investigations indicate that the patterns of enzyme activity, when used in conjunction with other assays, are useful in identifying distinct stages of lymphocyte maturation and differentiation.

The purpose of characterizing bovine CTL is to provide a system for studying the effects of bovine leukaemia virus (BLV) and other viruses on effector T-cell development and function. It was previously believed that BLV infected only B cells; however, recent reports indicate that it may also infect T cells (Lewin, Davis & Bernoco, 1985). Human retroviruses that infect T cells include HTLV-I, HTLV-II, and the agent associated with AIDS (Shaw *et al.*, 1984). Thus, BLV may be a useful animal model for the analysis of infection by a variety of retroviruses.

A shared structural feature that is unique to the genomes of HTLV-I, II and BLV is the presence of a large open reading

Abbreviations: ADA, adenosine deaminase; AK, adenosine kinase; APRT, adenine phosphoribosyl transferase; BLV, bovine leukaemia virus; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; dAK, deoxyadenosine kinase; dCK, deoxycytidine kinase; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; HGPRT or HPRT, hypoxanthine-guanine phosphoribosyl transferase; IL-2, interleukin-2; MLC, mixed lymphocyte culture; MoAb, monoclonal antibody; 5′-N, 5′-nucleotidase; NK, natural killer; PBL, peripheral blood lymphocytes; PNP, purine nucleotide phosphorylase; rh IL-2, recombinant human interleukin-2.

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frame which may regulate viral expression in infected cells (Ratner, Gallo & Wong-Stall, 1985). Because of the relation with HTLV, it is evident that BLV will be a useful animal model for the study of retrovirus infection. In order to study the effects of BLV on cell-mediated immunity in cattle, studies were initiated to characterize the cells involved. The purpose of the present studies was to determine whether the bovine mixed lymphocyte reaction could be used to generate cultures of CTL that could be maintained in rh IL-2. As will be shown in the present report, we have demonstrated that long-term cultures of CTL can be generated, characterized and maintained in rh IL-2.

MATERIALS AND METHODS

Isolation of cells

Blood from animals was collected by venepuncture into a sterile bottle containing glass beads. After constant agitation for 30 min, the blood was decanted from fibrin-coated beads and PBL prepared by density centrifugation using Ficoll–Hypaque as previously described by Rouse & Babiuk (1974).

Cell cultures

All cultures were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with L-glutamine and 10% heat-inactivated fetal calf serum (FCS) (Biolabs Inc.), streptomycin (100 u/ml) and penicillin (100 u/ml). Mixed lymphocyte cultures (MLC) were initiated in the following manner. Peripheral blood lymphocytes were isolated, as described above, and suspended in medium at a concentration of 2×10^6 cells/ml. One of the cell suspensions was exposed to 2000 rads X-irradiation. Equal volumes of the two suspensions were combined, and the culture was incubated at 37 in 5% CO_2. After 4 days, the culture was resuspended at 10^6 viable cells/ml (viability determined by trypan blue dye exclusion) and rh IL-2 (Chiron, Emeryville, CA; Biogen, Geneva, Switzerland) was added (100 units/ml). Cultures were maintained by adjusting the cell count to approximately 2×10^5 cells/ml with fresh medium containing rh IL-2 (100 units/ml) every 3 or 4 days. Cultured cells stained with Wright's stain were examined by light microscopy.

Cytotoxicity assay

Non-specific cytolytic activity was measured using a lectin-dependent assay employing YAC-1 cells as targets. Specific cytotoxicity was determined using concanavalin A (Con A)-activated stimulator bovine lymphocytes as targets. The lectin-dependent assay of Berke and co-workers was used with minor modifications (Berke, McVey & Clark, 1981). Briefly, 5×10^6 cells were pelleted and resuspended in 0·5 ml of DME with 10% FCS. To this, 50 μCi of $NA_2\ ^{51}CrO_4$ (1 mCi/ml New England Nuclear, Boston, MA) were added. The target cells were incubated for 60 min at 37 , then pelleted, washed in Hanks' balanced salt solution (HBSS), and resuspended in 10 ml of HBSS. After 45 min at 37 , the cells were again pelleted, counted and resuspended at a final concentration of 1×10^5 cells/ml in DME with 10% FCS. Cytotoxic measurements were conducted as follows. Triplicate 0·1-ml aliquots of the target cells and 0·1 ml of an aliquot containing various concentrations of effector cells suspended in medium were plated into round-bottomed microtitre plates (Limbro, Flow Labs, McLean, VA). In the lectin-dependent assay, 0·2 μg of Con A was added to the wells in 10 μl of medium. The plates were centrifuged at 200 ***g*** for 5 min and incubated at 37 in 5% CO_2. After 4 hr of incubation, 100 μl of supernatant were removed and the radioactivity of the sample determined. The percentage release was calculated as follows:

$$\% \text{ release} = \frac{(\text{c.p.m. experimental} - \text{c.p.m. spontaneous})}{(\text{c.p.m. total} - \text{c.p.m. spontaneous})} \times 100.$$

The spontaneous release was determined by incubating the targets with media alone. The total release was determined by incubating the targets with 1% Triton X-100. The spontaneous release was always less than 20%.

Surface markers

Cells were separated on Ficoll–Hypaque, subjected to several cycles of centrifugation and washing, and finally suspended in 0·1% gelatin in phosphate-buffered saline (PBS) containing 10^{-3} M azide. To evaluate the cell populations, 5×10^5 cells in 50 μl were added to 50 μl of the respective antibodies in 96-well round-bottomed microtitration trays and incubated for 30 min at 4 . The cells were washed four times and then treated with 50 μl of affinity purified fluoresceinated goat anti-mouse Ig, containing antibody to mouse light chains, and heavy chains of IgM, IgG, IgG2a, IgG2b, IgG3 (Tago, Burlingame, CA) for 30 min at 4 . The cells were washed three times and fixed in a 2% solution of formalin in PBS. The cell preparations were kept at 4 in the dark until examined.

The Becton-Dickinson FACS Research Analyser model 420 and Consort 30 computer were used to analyse the patterns of reactivity of MoAb with the cells. Two-parameter analysis, wide-angle scatter versus fluorescence, was used. The data were recorded as two-parameter contour plots and the percentage labelled cells determined by the Consort 30 statistics program provided by the Becton-Dickinson FACS Systems Division.

Monoclonal antibodies

The MoAb used in the present study to identify subpopulations of bovine lymphocytes were selected from a panel of 70 MoAb currently under investigation in our laboratory. Complete characterization will be published elsewhere. Briefly, the general characteristics are described herein. A study is in progress to develop a set of MoAb that react with conserved determinants on phylogenetically related leucocyte differentiation antigens and antigens determined by the major histocompatibility complex for use in comparative studies in multiple species (Davis, Perryman & McGuire, 1984; Davis & Maurice, 1985). The accumulated data on the antibodies used here are listed in Table 1. The antibodies to MHC antigens detect highly conserved determinants expressed on cells in many species. H58A detects a monomorphic determinant in most of the species thus far examined. The determinant is polymorphic in pigs and mice. Analysis of 45 inbred strains of mice has shown that the antibody reacts with the H-$2K^{f,p,pr}$ and H-2^{w16} allelic gene products. H42A reacts with H-2^{w3} and TH81A5 reacts with H-$2^{b,f,r,s,u,v,w4,w6,w7,w17,w23}$. Preliminary comparative studies with human cells indicate that anti-class II antibodies detect three molecules corresponding to DP (H42A), DR (TH14B) and DQ (TH81A5). Immunoprecipitation and SDS/PAGE autoradiographic studies show that the antibodies react with molecules with molecular weights corresponding to class I and class II antigens. Sequential absorption of lysates of bovine leucocytes has shown that H42A, TH14B and TH81A5 react with separate

Table 1. Monoclonal antibodies for analysis of the major histocompatibility gene complex and the immune system in ruminants

MoAb no.	Ig isotype	% peripheral blood leucocytes positive for antigen						Apparent specificity
		Cow	Sheep	Goat	Pig	Horse	Human	
H58A*	IgG2a	95	94	85	83	99	95	MHC-class I
H42A†	IgG2a	20–28	20–29	11–20	28	32–59	19	MHC-class II
TH14B	IgG2a	20–30	30–33	34	—‡	20–41	20	MHC-class II
TH81A†	IgG2a	30(P)	22–30	15–40	30	—	14	MHC-class II
B26A	IgM	30–50	—	36	—	—	—	Pan T
CH128A	IgM	25–40	—	—	—	—	—	Pan T
CH127A	IgM	35	27	28	—	—	—	T subset
DH59A	IgG1	20–26	15–20	20–26	20	10	15–19	Monocyte + granulocyte
PIg45A	IgG2b	15	12–21	10–17	10–17	12–17	—	IgM

* Antigen polymorphic.
† Reacts with mouse leucocytes.
‡ Negative.

molecules. B26A and CH128A are pan T antibodies that react with the bovine sheep red blood cell receptor and completely block rosetting; interspecies cross-reactivity, however, shows that B26A and CH128A react with different determinants (Table 1). Analysis by dual fluorescence labelling of Hypaque–Ficoll purified, thrombin-treated preparations of peripheral blood leucocytes has shown that B26A reacts with 90% of the surface Ig-negative cells in T-enriched preparations of lymphocytes (Lewin *et al.*, 1985). CH127A reacts with a subpopulation of cells with a low expression of the sheep red blood cell receptor. It is not yet clear how this population relates to T-helper and T-suppressor cells. PIg45A reacts with IgM in multiple species (Davis *et al.*, 1984) and has an identical pattern of reactivity with an anti-IgM MoAb described by Donahoe *et al.* (1984). DH59A detects a determinant present on granulocytes and monocytes. Comparative studies by fluorescence microfluorimetry have shown that the antibody detects a molecule with a similar or identical distribution with one detected with Leu M1 (Hanjan, Kearney & Cooper, 1982; Davis *et al.*, 1984).

Enzyme assays

The activities of adenosine kinase (AK), 2′-deoxyadenosine kinase (dAK), 2′-deoxycytidine kinase (dCK), 5′-nucleotidase (5′-N), purine nucleotide phosphorylase (PNP), adenosine deaminase (ADA), adenine phosphoribosyl transferase (APRT), hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), and AMP deaminase were measured using a modified microassay originally described for human PBL (Van Laarhoven *et al.*, 1980). In brief, 3 μl of a lymphocyte suspension containing 7×10^7 cells/ml in 0·9% NaCl were pipetted into small incubation vessels prepared from Parafilm. The cells were frozen at −20 for 30 min and subsequently lyophilized. The appropriate substrate was added to the lyophilized cells and allowed to incubate. The enzyme activities, except for the kinases, were quantified by separating products and substrates by thin-layer chromatography. The kinase activities were quantified by separating products and substrates on ion exchange filters. All buffers, substrates, incubation times and chromatographic conditions are as previously reported (Magnuson *et al.*, 1985). Enzyme activities were linear for up to twice the assay times used.

RESULTS

Establishment of cell cultures

Peripheral blood lymphocytes from one animal were X-irradiated and a MLC initiated by mixing cells from one animal with the X-irradiated cells from the second animal. After 4 days, the cultures were centrifuged and suspended in medium containing rh IL-2. Maintenance of the culture depended upon the continued presence of IL-2. Under these conditions, the cultures usually thrived for 2 months before growth subsided. Occasionally cultures would maintain vigorous growth for as long as 6 months before encountering the first growth crisis [a typical observation for lymphocytes of other species (Grimm & Rosenberg, 1982)]. The morphological features of the cells in long-term culture, as determined by Wright staining, changed from small resting lymphocytes to large granular lymphocytes (Fig. 1). The morphology of the cultures remained unchanged after the first several weeks in culture.

Development of cytotoxic activity by the cell cultures

The cultures were tested at various times for their ability to lyse YAC-1 cells in a lectin-dependent assay. It has been reported that CTL kill in both an antigen-specific fashion and in lectin-dependent assays *in vitro* (Bradley & Bonavida, 1981). It has also been suggested that the mechanism of killing is the same in both cases (Berke, 1983). For these reasons, we used the lectin-dependent assay to measure the total cytolytic activity of our cultures. The data presented in Table 2 indicate that cytolytic activity was low after 1 week (6% or less). A steady increase in cytolytic activity occurred during the second and third weeks, reaching values of 50% or greater. The pattern for the gradual development of cytotoxic activity was a consistent feature, although the actual time period for the development of

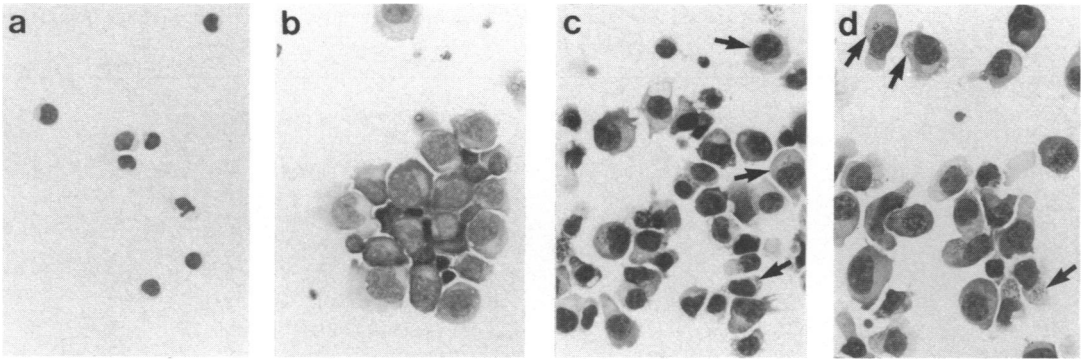

Figure 1. Morphology of peripheral blood cells following stimulation in an MLR and incubation with recombinant human IL-2: (a) fresh cells; (b) cells in culture 7 days; (c) cells in culture 18 days; (d) cells in culture 5 weeks. The arrows indicate several of the cells which have typical LGL morphology.

Table 2. Development of cytolytic activity in bovine T lymphocyte cultures maintained in recombinant human IL-2 (percentage ^{51}Cr release*)

	Days in culture																								
	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Exp. 1†		<5%													50										76
Exp. 2	<5%						14														43				
Exp. 3		6								20			66												

* The cytotoxicity was determined as reported in the Materials and Methods. All data represent E:T ratios of 20:1 in the presence of Con A (0·2 μg/well).

† Each experiment represents MLCs initiated on different days using cells from the same animals and the same culturing protocols.

cytotoxic activity varied from culture to culture. Furthermore, the magnitude of killing was observed to fluctuate (45–80%) in cultures maintained for 3–6 weeks for reasons which are not understood at this time.

Specific cytotoxicity

The data presented in Table 3 show that cultures 2–3 weeks old exhibit specific cytotoxic activity for Con A blasts from the animal that served as a source of stimulator cells in the MLC (90% release). The fact that these cultures did not lyse Con A-generated blasts from a different animal or self (less than 10%, data not shown) demonstrates that the killing is specific. After 5 weeks, the cultured cells lost their ability to lyse the specific target in the absence of lectin.

Table 3. Demonstration of specific cytolytic activity

		^{51}Cr release	
Effector cell	Effector:target*	(+) Con A†	(−) Con A
19-day MLC‡	30:1	84	90
	10:1	66	82
	3:1	38	
	0·3:1	7	10
49-day MLC	50:1	84	<1
	25:1	74	<1
	12:1	54	<1
	1:1	11	<1

* The targets used are Con A-stimulated peripheral blood lymphocytes from the same animal which provided stimulator cells for the MLC. The cells had been maintained for 2 weeks in culture in media containing rh IL-2.

† Concentration of Con A used was 1 μg/ml.

‡ Assay was carried out in triplicate as described in Materials and Methods.

Surface markers

The increase in cytotoxic activity over the first several weeks of culture with rh IL-2 suggested that a specific subpopulation of lymphocytes were preferentially growing in the culture. In order to monitor the phenotype of this cell population, monoclonal antibodies that identify bovine cell surface antigens were used (Davis *et al.*, 1984; Lewin *et al.*, 1985). The data in Table 4 indicate that cells belonging to the T-cell lineage were the predominant cell in the long-term cultures. This is shown by the decrease in the number of cells positive for class II antigen and surface IgM (B-cell markers) and by the increase in the numbers of cells reactive with MoAb specific for T cells. The cell population in the long-term cultures was still heterogenous as shown by the light scatter and staining characteristics. The largest population of cells was strongly positive for B26A and CH128A, and exhibited high light-scattering characteristics. Analysis by volume versus fluorescence showed that these cells correspond to the large blast cells. A small population comprising cells with low light-scatter exhibited low levels of expression of B26A and CH128A and high levels of CH127A. Reactivity with DH59A, H42A, TH14B and TH81A was below background. H58A reacted with all cells as expected.

Table 4. Surface markers on bovine lymphocytes maintained in continuous culture

Monoclonal antibody		% stained			
		Day 8	Day 10	Day 18	Day 33
Class I*	(H58A)	96·2	88·1	ND†	83·9
Class II	(H42A)	33·5	33·2	6·2	9·0
	(TH14B)	37·2	34·8	2·7	9·3
	(TH81A)	19·0	13·6	ND	ND
Pan T	(B26A)	54·8	59·0	88·2	90·0
	(Ch128A)	50·5	57·8	ND	86·8
T subpopulation	(CH127A)	ND	10·8	20·3	10·8
Macrophage and granulocytes	(DH59B)	9·6	ND	0	8·6
IgM	(PIg45A)	9·6	ND	8·7	6·7
Background	(2nd Ab)	7·6	2·7	4·3	7·5

* Specificity of monoclonal antibodies.
† ND, not determined.

Table 5. Enzyme activities in bovine lymphocytes

	Resting	Long-term
AK*	0·67 ± 0·14† (3)‡	0·30 ± 0·04 (3)
5′-nucleotidase		
AMP§	0·477 ± 0·062 (5)	1·9 ± 0·5 (3)
deoxyAMP§	0·366 ± 0·113 (5)	2·08 ± 0·39 (5)
PNP	0·752 ± 0·122 (3)	6·52 ± 0·62 (3)
ADA		
Ado§	28·6 ± 1·3 (4)	20·4 ± 3·9 (3)
deoxyAdo§	14·6 ± 0·2 (4)	ND¶
APRT	2·17 ± 1·63 (6)	5·73 ± 1·34 (4)
HPRT	0·483 ± 0·031 (6)	4·45 ± 0·47 (4)
AMP deaminase	50·5 ± 8·2 (4)	80·8 ± 9·4 (3)

* No activity was detected for dAK or dCK.
† Activities are in nmole/(hr × 10^6 cells) ± SE.
‡ Indicates the number of animals or cultures tested.
§ Substrates.
¶ ND, not determined.

Enzyme activities

In an attempt to determine whether any of the purine metabolic enzymes could serve as markers for long-term IL-2-dependent cells, the activities of several enzymes in fresh PBL and lymphocytes grown in long-term culture were compared. The enzyme activities are shown in Table 5. The activities of many of the enzymes increased in the cells grown in long-term cultures. Most notable were the activities for HPRT and PNP for which the activity increased almost 10-fold over fresh cells. A four-fold increase in activity was observed for 5′-N and AK. A small increase in the activity for AMP deaminase was observed, while the activity of ADA decreased.

DISCUSSION

We report here the successful establishment of bovine CTL with rh IL-2 after a primary *in vitro* alloantigen challenge. A gradual development (several weeks) of specific and non-specific cytolytic activity was consistently observed. The reason for this slow development of CTL is not clear; however, it is known that suppressor cells are present after a primary MLC (Truitt, Rich & Rich, 1977; Susskind *et al.*, 1983). These suppressor cells may play a role in blocking the development of the CTL precursors (Susskind *et al.*, 1983). Alternatively, the lack of cytolytic activity for cells in culture less than 2 weeks may reflect a low number of effector cells which were not detectable with our assay conditions.

Using two different assay systems, it was possible to monitor both specific and non-specific cytolysis. Natural killer cells have been identified in other species (Ortaldo & Herberman, 1984) and have been implicated as being important in non-specific killing as a first defence against virus infection and tumour growth. The observation that specific cytotoxicity was lost when CTL were maintained in long-term culture is consistent with the work of Brooks *et al.* (1983) who reported that NK activity appeared in cloned CTL cell lines grown in long-term culture. It has been hypothesized that the loss of specific cytotoxicity may represent a differentiation of CTL to an NK-like cell (Brooks *et al.*, 1983). Even though NK cells and cells that participate in lectin-dependent cytotoxicity are distinct populations (Bradley & Bonavida, 1983), NK cells can also kill a NK-insensitive target in the presence of lectin (Bonavida, LeBow & Bradley, 1984). The morphology observed in our cultures is similar to that associated with NK cells in human and rat systems (Ortaldo & Herberman, 1984). Moreover, clones of NK cells express the sheep red blood cell receptor (Hansen *et al.*, 1984). On the other hand, the loss of specific cytotoxicity may reflect a shift in the population of cells from CTL to other T cells. This latter hypothesis has not yet been tested because assays for NK cell activity in cattle have not yet been clearly defined (Campos & Rossi, 1983; Bielefeldt-Ohmann, Davis & Babiuk, 1985). Other possibilities for loss of specific cytotoxicity may exist. Cells without specificity may expand at a greater rate than alloantigen-specific CTL. Restimulation was not examined in these studies but might enhance outgrowth of alloantigen-specific CTL (Picha & Baker, 1986).

Monoclonal antibodies, specific for bovine cell surface

antigens, demonstrated that cell populations do change in our cultures. The availability of a large panel of MoAb has proven useful in several laboratories in identifying cell populations (Davis *et al.*, 1984; Bielefeldt-Ohmann *et al.*, 1985; Lewin *et al.*, 1985). Several of these MoAb reveal shifts in the composition of cell populations. Our data show that there is a progressive increase in pan T-positive cells during long-term culture, and a corresponding decrease in class II surface IgM-positive cells. This is consistent with reports that T-helper, NK and CTL populations proliferate and expand in the presence of IL-2 (Gillis & Smith, 1977; Smith, 1980).

Large differences in activities of purine metabolic enzymes have been found in lymphocytes from various species (Barton & Goldschneider, 1979; Barton *et al.*, 1980; Carson, Kaye & Wasson, 1980; Peters & Veerkamp, 1983). In addition to these differences, different patterns of enzyme activities occur between many lymphocyte subpopulations (Ma *et al.*, 1982; Barton *et al.*, 1980). In man, for example, 5′-N is high in suppressor cells and low in helper T cells (Thompson *et al.*, 1983). In immature T lymphocytes, both PNP and 5′-N are low but increase as the cells mature (Ma *et al.*, 1982). Adenosine deaminase activity, on the other hand, decreases as cells mature (Ma *et al.*, 1982). In our cultures all the enzyme activities increased, with the exception of ADA which decreased. This finding was consistent with observations for cultured human IL-2-dependent CTL (Marianne, Castellazzi & Buttin, 1984). The two enzymes which increased the most were HPRT and PNP. These findings suggest that the pattern of low ADA activity with high HPRT and PNP activities may be an additional identification marker for CTL in cattle.

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