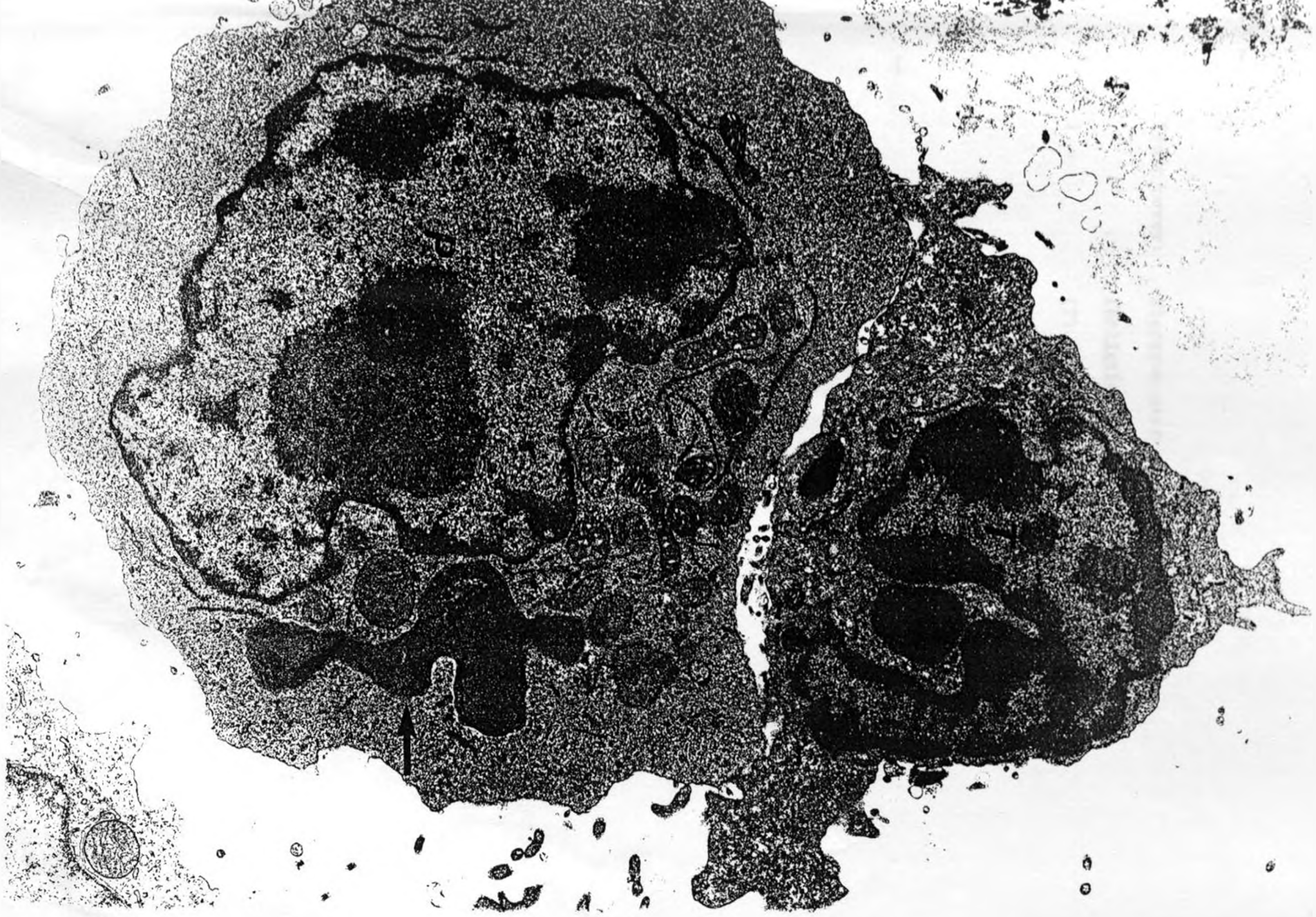

Front cover: Electron micrograph showing a *Theileria*-specific cytotoxic T cell (T) attached to the surface of a parasitized lymphocyte (P). The arrow indicates the *Theileria* schizont in the parasitized cell.

Back cover: Electron micrograph showing a lysed parasitized lymphocyte (P) after contact with a *Theileria*-specific cytotoxic T cell (T). The arrow indicates a *Theileria* schizont in the lysed parasitized cell.

(Photos and captions courtesy of Drs. B.M. Goddeeris and S. Ito.)

PARASITE SPECIFITY OF CYTOTOXIC T CELLS FROM CATTLE IMMUNIZED WITH BUFFALO AND CATTLE-DERIVED *THEILERIA PARVA* PARASITES

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A thesis submitted in partial fulfilment for the degree of Master of Science of the University of Nairobi

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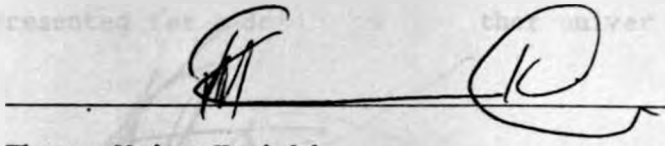
1989

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.



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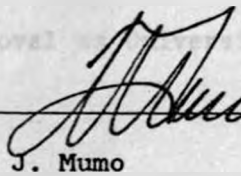

Dr. J. Mumo

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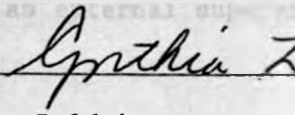


Dr. C. L. Baldwin

ABSTRACT

Immunity to East Coast fever, a parasitic disease of cattle, is partially attributable to a cell-mediated immune response directed at cells infected with the schizont stage of the parasite. This response is in the form of cytotoxic T cells which can be measured *in vitro* after stimulating peripheral blood mononuclear cells (PBM) from immune animals (but not from naive animals) with their own autologous *Theileria parva*-infected cells. This study evaluated the parasite strain specificity of cytotoxic cells generated in cattle immunized with one of 5 different stocks of buffalo-derived *T.parva lawrencei* parasites or with cattle-derived *T.p.parva* (Uganda) or *T.p.bovis* (Boleni) parasite stocks. The objective was to determine whether any of these stocks was able to generate non-strain restricted immune T cells, that would recognize and subsequently lyse lymphocytes infected with different strains of *T.parva* parasites. Ultimately such information would be useful in identifying an antigen on the surface of schizont-infected cells which is common among cell lines infected with many different strains or stocks of *T.parva* parasites, and which could potentially form the basis of a recombinant subunit vaccine against East coast fever.

To do this a group of 7 embryo transfer cattle, sharing the same parents and matched for at least one bovine lymphocyte antigen (BoLA) A-locus specificity, were immunized by infection and treatment. Cell lines to be used as target cells in *in vitro* cytotoxicity assays were generated by infection of PBM with the 5 buffalo parasite stocks as well as with the cattle-derived parasite stocks *T.p.parva* (Uganda), (Muguga), (Marikebuni), (Mariakani), and *T.p.bovis* (Boleni). PBM from the 7 cattle were evaluated in cytotoxicity assays following repeated stimulation *in vitro* with autologous or major histocompatibility complex (MHC)-matched cells infected with the same parasite stock as was used for immunization of the animal. It was possible to generate cytotoxic cells from all 7 cattle which were specific for MHC-matched cells infected with *T.parva* parasites. Testing for MHC restriction specificity using a panel of lymphocytes of different MHC backgrounds but infected with a single *T.parva* parasite stock were carried out and the results indicated bias in response towards one of the 2 BoLA A-locus class 1 specificities of the donor animals. In addition, limiting dilution assays for the estimation of the frequency of precursors of cytotoxic T cells with various parasite specificities in the blood of the immune cattle were carried out for 5 of the animals. The results in terms of parasite specificity of cytotoxic cells were very similar to those obtained with *in vitro* cytotoxicity assays.

In summary, these experiments have revealed that the buffalo-derived parasite stocks Tp1(7014), (7013), (6999), (7065), (6998) and the cattle derived Tpp(Uganda) stock were able to induce generation of cytotoxic T-cells *in vivo* which are capable of recognizing parasite antigens on cells infected with many different *T.parva* parasite stocks although the proportion of cells killed was low for some of the cell lines. Further, lymphocytes from 1 steer which had cytolytic activity against a large number of infected cell lines were cloned in order to determine if individual immune T-cells were recognizing antigenic epitope(s) common among cells infected with different stocks of *T.parva*, or if the broad specificity of killing was due to a heterologous mix of cytotoxic cells with varying antigen specificities. Two of the T cell cytotoxic clones generated gave greater than 50% cytotoxicity on all of the infected target cells at an effector to target ratio of less than 5:1. Such cloned cells will be very useful in future studies designed to identify the common or cross-reactive antigenic epitope and the parasite gene encoding for it, with a view towards constructing a recombinant vaccine against East coast fever.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my external supervisor Dr. C.L. Baldwin for her advice, encouragement, inspiration and patience during the course of this work and for reviewing this manuscript. She went much beyond her required duties in providing her time and personal interest.

I would also like to thank my university supervisor Dr. J. Mumo for helpful discussions and continuous interest during the course of this work. Many thanks are also due to Drs. T.T. Dolan, J.G. Grootenhuis, B.M. Goddeeris, W.I. Morrison, T. Jordt, E.Taracha, S.Morzaria and A.J. Teale for helpful discussions and supply of material necessary to conduct this work. I am grateful to Ferdinand Mbwika, Reeves Njammungeh, Joel Mwakaya and Elias Awino for providing technical assistance and to Joel Mwaura and Francis Shikubari for skillful assistance with graphic arts. I wish to express my special thanks to Mary Mbaabu for assistance in preparation of this manuscript and to Catherine for helping ease the struggles.

I gratefully acknowledge the opportunity granted to me by the Directors of the International Laboratory for Research on Animal Diseases (ILRAD) where this work was conducted and Dr. J. Lenahan, training officer, ILRAD.

I was sponsored by the German Academic Exchange Agency (DAAD) through the University of Nairobi and am greatly indebted to them for making this possible.

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

GENERAL:

BoLA	Bovine lymphocyte antigen
Con A	Concanavalin A
CPM	Counts per minute
CTL	Cytotoxic T-Lymphocyte(s)
DMSO	Dimethyl sulphoxide
ECF	East Coast fever
E:T	Effector:target ratio
FCS	Foetal calf serum
GBq	Giga Becquerels
IFA	Immunofluorescence assay; Immunoflourescence antibody test
ILRAD	International Laboratory for Research on Animal Diseases
^{111}In	[^{111}In]Indium oxine
IUDR	[^{125}I]Iododeoxyuridine
LDA	Limiting dilution assays
MAb	Monoclonal antibody
2ME	2-Mercaptoethanol
MHC	Major histocompatibility complex
MLC	Mixed leukocyte culture
MLR	Mixed leukocyte reaction

PBM	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PHA	Phytohaemagglutinin
TCGF	T-cell growth factor
TCMN-LA	Tetramycin injectable solution-long acting

SUBSPECIES NOMENCLATURE:

Tpb	*Theileria parva bovis*
Tpl	*Theileria parva lawrencei*
Tpp	*Theileria parva parva*

PARASITE STOCKS:

Tpb (Boleni)	The Boleni stock of Tpb
Tpp (Mariakani)	The Mariakani stock of Tpp
Tpp (Marikebuni)	The Marikebuni stock of Tpp
Tpp (Muguga)	The Muguga stock of Tpp
Tpp (Uganda)	The Uganda stock of Tpp
Tpl (6998)	The stock of Tpl derived from buffalo no.6998
Tpl (6999)	The stock of Tpl derived from buffalo no.6999
Tpl (7013)	The stock of Tpl derived from buffalo no.7013
Tpl (7014)	The stock of Tpl derived from buffalo no.7014
Tpl (7065)	The stock of Tpl derived from buffalo no.7065

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 THE PARASITE: CLASSIFICATION, OCCURRENCE AND EPEDEMIOLOGY

The protozoan parasite Theileria parva is the causative agent of the cattle disease commonly known as East Coast fever (ECF) or East African theileriosis. The parasite is transmitted by the three host tick, Rhipicephalus appendiculatus. According to the recently revised classification of protozoa (Levine et al., 1980),the parasite is classified as follows:

Subkingdom: Protozoa
Phylum : Apicomplexa
Class : Sporozoea
Subclass : Piroplasmia
Order : Piroplasmida
Family : Theileriidae
Genus : Theileria
species : parva

In addition three tentative subspecies of T.parva have been identified for convenience rather than scientific accuracy on the basis of clinical, epidemiological and behavioral characteristics. They are T.parva parva and T.p bovis, both derived from cattle, and T.p lawrencei, parasites derived from buffalo (Uilenberg, 1981).

East Coast fever is endemic in a large area of East and Central Africa where it is the second major killer of livestock

and a major hindrance to the expansion and improvement of the livestock industry (Duffus, 1976). Between 50 and 80% of the estimated 10 million cattle in Kenya are exposed to the tick vector and approximately 1% of these die of ECF annually (Duffus, 1976). In susceptible cattle exposed to infection with T.parva, morbidity and mortality are approximately 100% and 95% respectively (Brocklesby et al., 1961).

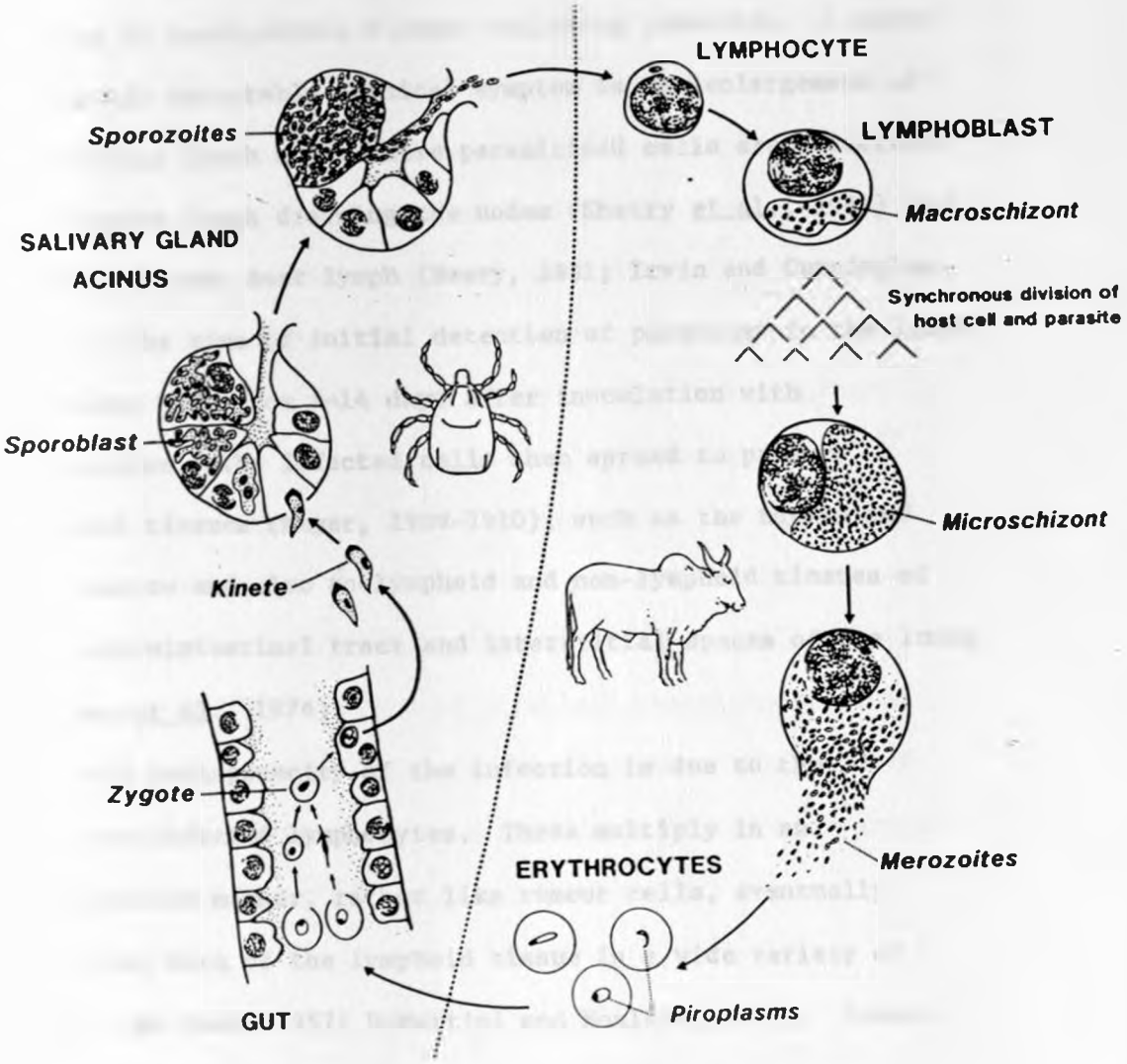
The incidence and prevalence of the disease closely follows the distribution of R.appendiculatus, which is found in areas of high rainfall (greater than 400 mm/yr) and where conditions are warm, humid and with a thick grass vegetation (Neitz, 1957; Irvin and Cunningham, 1981). Another important factor in the epidemiology of the disease is the African buffalo Syncerus caffer, an asymptomatic carrier of the infection (Barnett and Brocklesby, 1966) which acts as a reservoir of parasites which may be maintained and transmitted by the tick population to cattle.

1.2 LIFE CYCLE

The three-host tick R.appendiculatus transmits T.parva transtadially. Infection is acquired by the larvae or nymphal stages and transmitted following moulting by the nymphal or adult stages of the tick (Theiler, 1904). Ticks become infected with T.parva following a blood meal from cattle or buffalo that carry the piroplasm stage of the parasite in their blood. The infected red blood cells release male and female

gametes in the gut and these fuse to form a zygote that invades the gut wall several days after the ticks have completed feeding and detached from the host (Gonder, 1911; Schein et al., 1978). The tick moults and during and following this process motile kinetes released from the gut enter the haemolymph, then migrate to the salivary glands and invade the E cell of the type III acini (Young and Leitch, 1980; Fawcet et al., 1982; Binnington et al., 1983). Mehlhorn and schein (1984) describe the infection of both D and E cells of type III acinar cells. Here they undergo sporogony to produce a mass of sporozoites (ramifying syncitium) whose processes occupy most of the greatly enlarged glandular cell (Fawcett et al. 1982, 1985). Sporogony only occurs when the tick commences feeding. Sporozoites in the tick saliva are injected into the mammalian host during feeding (Stagg et al.(1981). Fawcett et al. (1985) from studies of T.parva and T.taurotragi has shown that in bovine hosts the sporozoites invade lymphocytes by an endocytosis then undergo nucleic division to produce multinucleate schizonts as a single ramifying syncytium, but Mehlhorn et al, (1979) and Mehlhorn and Schein, (1984) describe the formation of multiple sporoblasts and cytomeres from studies of many species. Division of host cell and schizont occurs synchronously (Stagg et al., 1980) resulting in a rapidly expanding population of parasitized cells (Hulliger et al., 1964). A proportion of the schizonts later give rise to merozoites (Schein et al., 1978). These then enter erythrocytes and develop into piroplasms. A diagram of the life cycle is presented in Fig. 1.

Fig. 1 Life cycle of Theileria parva. The left-hand side of the cycle represents the development in ticks while the right-hand side represents the development in cattle. (Reproduced by kind permission of Dr. B. Godderis, ILRAD, Nairobi, Kenya).



Life cycle of ***Theileria parva***

1.3 THE DISEASE

The pathology of the disease caused by T.parva parasites has been extensively reviewed by Irvin and Morrison (1987). East Cost fever is a fatal disease which in susceptible cattle results in death within 3 weeks following infection. A major and easily detectable clinical symptom is the enlargement of superficial lymph nodes where parasitized cells are detectable in efferent lymph draining the nodes (Shatry et al., 1981) and in the thoracic duct lymph (Emery, 1981; Irvin and Cunningham, 1981). The time of initial detection of parasites in the lymph nodes may vary from 4–14 days after inoculation with sporozoites. The infected cells then spread to primary lymphoid tissues (Meyer, 1909-1910), such as the thymus and bone marrow and also to lymphoid and non-lymphoid tissues of the gastrointestinal tract and interstitial spaces of the lungs (Radley et al., 1974).

The pathogenecity of the infection is due to the schizont-infected lymphocytes. These multiply in an uncontrolled manner, rather like tumour cells, eventually replacing much of the lymphoid tissue in a wide variety of organs (De Kock, 1957; DeMartini and Moulton, 1973). Severe pulmonary oedema (Cowdry and Danks, 1933) and respiratory distress are characteristic of the terminal stages of the disease (Theiler, 1904; reviewed by Irvin and Morrison, 1987). The later stages of the disease are characterized by widespread lymphocytolysis with profound depletion of lymphocytes both in

the solid lymphoid tissue and in the recirculating pool (Wilde et al., 1966; Morrison et al., 1981). Other detectable changes include a complete atrophy of normal lymphoid elements (Steck, 1928), replacement of normal bone marrow hemopoietic tissue by parasitized cells and ulceration of abomasun and the small intestines. Although the mechanisms of lymphocytolysis are not compretely understood, the widespread destruction of lymphoid and non-lymphoid tissues are not believed to be due solely to infection with the parasite (Morrison et al., 1986a). There is no evidence for antibody-mediated lytic mechanisms (Duffus et al., 1978; Creemers, 1982) but cytolytic cells have been demonstrated in the peripheral blood of cattle undergoing lethal infections (Emery et al., 1981). There is also activation of complement (Maxie et al., 1982), with release of vasoactive compounds which may be of particular significance in the development of pulmonary oedema (Shitakha et al., 1983). Although significant levels of piroplasm parasitaemia (over 50%) are detectable in infected animals, there is only mild anaemia which is not of major significance in the pathogenesis of the disease (Morrison et al., 1986a).

1.4 PREVENTION AND TREATMENT OF EAST COAST FEVER

There are three principle ways of controlling ECF. The first is prevention of the disease. This is largely dependent upon control of ticks by regular spraying or dipping of cattle with acaricides. This is expensive and in some areas where

dipping must be carried out as often as twice a week, the costs are prohibitive. An additional problem is the development of tick resistance to certain acaricides. The dipping strategy is widely applied especially in areas where costs are considerably defrayed by introducing augmentative measures such as restriction of livestock movement and where contact between carrier buffalo and susceptible cattle is minimized by fencing (Morrison et al., 1986a).

A second method of control is immunization against T.parva infections. This is done either by simultaneous injection of the parasite stabilate and long acting oxytetracyline which limits parasite growth in vivo or by giving low doses of the parasite which the animal is able to control (Dolan et al., 1984). Immunization has a number of practical limitations as outlined by Radley et al. (1975a). Immunization with one strain may only protect against the homologous strain but not heterologous strains. The use of a "cocktail" of parasite strains for immunization, despite its widespread applicability, has certain drawbacks, the major one being that the animals may become asymptomatic carriers of infection. The method of infection and treatment, that is, chemoprophylaxis and stabilate inoculation has however been used successfully (Dolan et al., 1980).

The third method is treatment of cattle that become infected with T.parva parasites. In the last decade two therapeutic compounds have been developed that are effective against T.parva: parvaquone (Clexon, Wellcome) and halofuginone

(Terit, Hoechst) (Dolan, 1981). These compounds are of considerable value in the treatment of affected cattle, although the effective use requires early diagnosis of the disease. Their use also involves considerable expense.

1.5 HETEROGENEITY OF PARASITE STOCKS

The number of strains of T.parva parasites that occur in the field is not known but is believed to be limited (Morrison et al., 1986a). The existence of multiple strains is a major hindrance to the exploitation of currently available methods of immunization by infection and treatment. Many examples exist of a parasite stock that protects against challenge with another stock but not vice versa (Young et al., 1973; Radley et al., 1975a). For instance immunization against one strain of T.p.parva will protect against the immunizing strain but may not protect against T.p.lawrencei parasites (Cunningham et al., 1974; Radley et al., 1975b). Even strains of T.parva common among cattle may not cross-protect. For instance cattle immunized with T.p.parva (Muguga) are susceptible to challenge with T.p.parva (Marikebuni). Conversely, animals immunized against T.p.parva (Marikebuni) are protected against both the immunizing strain and T.p.parva (Muguga) (Radley et al., 1975b; Irvin et al., 1983). Furthermore, animals immunized against T.p.lawrencei are frequently protected against a broader spectrum of parasite stocks including both T.p.lawrencei and T.p.parva (Young et al., 1973; Dolan et al.,

1980). A 'cocktail' containing two stocks of T.p.parva and one stock of T.p.lawrencei showed significant protection in laboratory immunization trials (Radley et al., 1975a), although animals immunized with the cocktail, exhibited breakthrough infections when challenged with another stock of T.p.lawrencei (Radley et al., 1979).

It is evident that the problem of parasite heterogeneity may be particularly important in areas where the buffalo reservoir and T.p.lawrencei are prevalent. Since buffalo are asymptomatic carriers of infection it is probable that multiple strains of the parasite will most frequently be generated in them and transmitted by ticks to susceptible cattle hosts. The T.p.lawrencei parasites produce a pathogenic reaction in cattle with low numbers of schizonts and piroplasms while T.p parva produces high numbers of these stages. When T.p.lawrencei is passaged through ticks and cattle, the behavioral pattern of the parasite changes to produce high numbers of schizonts and piroplasms (Barnett and Brocklesby, 1966). This indicates the antigenic diversity or variation of T.p.lawrencei parasites that is possible within the buffalo host. The genomic diversity of these parasites is detectable by DNA probes (Conrad et al. 1987a).

1.6 IMMUNITY

Cattle that recover from ECF spontaneously are immune to subsequent challenge with T.parva parasites and they can be

immunized by the method of infection and treatment described above. Immunity is at least partially attributed to a cell-mediated immune response directed at the schizont stage of the parasite (Pearson *et al.*, 1979). The response is in the form of cytotoxic T-cells. These can be generated *in vitro* by stimulating peripheral blood mononuclear cells (PBM) from immune animals, but not naive animals, with their own autologous *T.parva*-infected cells (Pearson *et al.*, 1979, 1982). The cytotoxic T-cells have been shown by Goddeeris *et al.* (1986a) to bear the surface marker $BoT8^+$ which is identified by monoclonal antibody (MAb) IL-A17 (Ellis *et al.*, 1986).

Other *in vitro* studies (Goddeeris *et al.*, 1986b; Morrison *et al.*,1986b; Teale *et al.*, 1986) have shown that cytotoxic cells will kill their own (autologous) cells infected with *T.parva* as well as infected cells from different animals (allogeneic cells) which are matched or partially matched for major histocompatibility complex (MHC) antigens called bovine lymphocyte antigens (BoLA) (Fig. 2). It is believed that the cytotoxic T-cells recognize parasite-induced antigenic changes on the surface of infected cells in conjunction with self MHC antigens and subsequently lyse the infected cells (Goddeeris *et al.*, 1986a), which is similar to the phenomenon of lysis of virus-infected target cells by cytotoxic T-cells in mice and man (Zinkernagel and Doherty, 1979) (Fig. 3). The BoLA molecules recognized by $BoT8^+$ cells are coded for at the A locus of the MHC gene complex and are known as class I MHC

Fig. 2 Genetic restriction of cytotoxic T cells showing how killer T cells recognize parasite induced antigens (open triangle) on the surface of T.parva-parasitized cells in conjunction with self MHC antigens (open square) and subsequently lyse them, but do not lyse parasitized cells bearing non-self MHC antigens (open circle). (Reproduced by kind permision of Dr. W. Ivan Morrison, ILRAD, Nairobi, Kenya).

Genetic restriction of Cytotoxic T cells

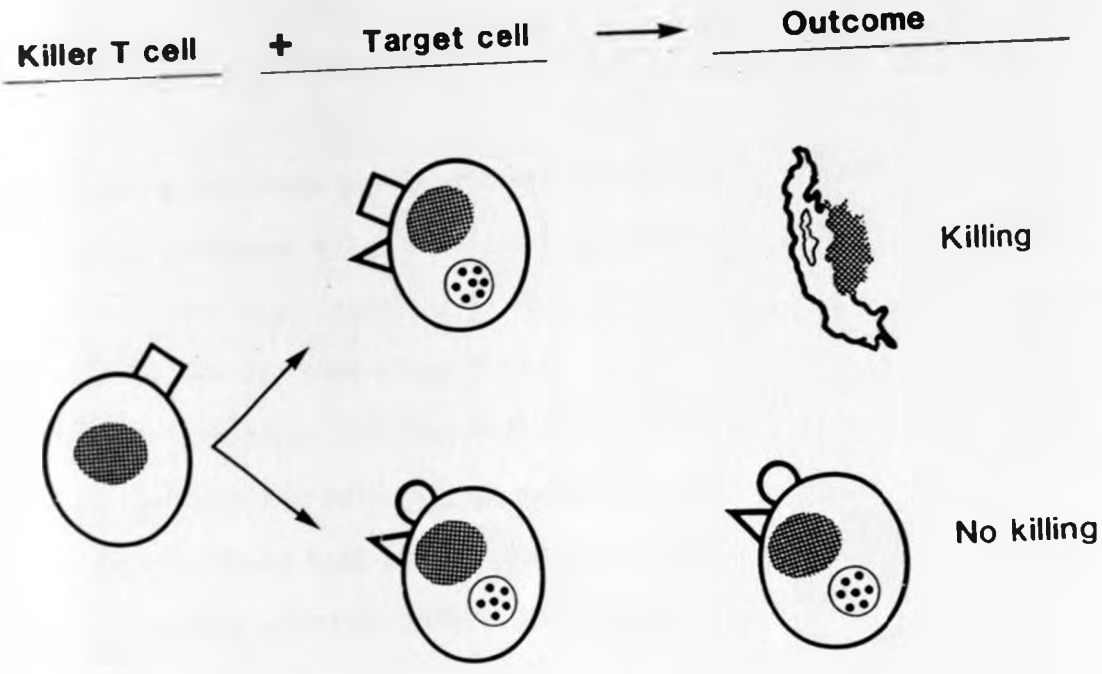
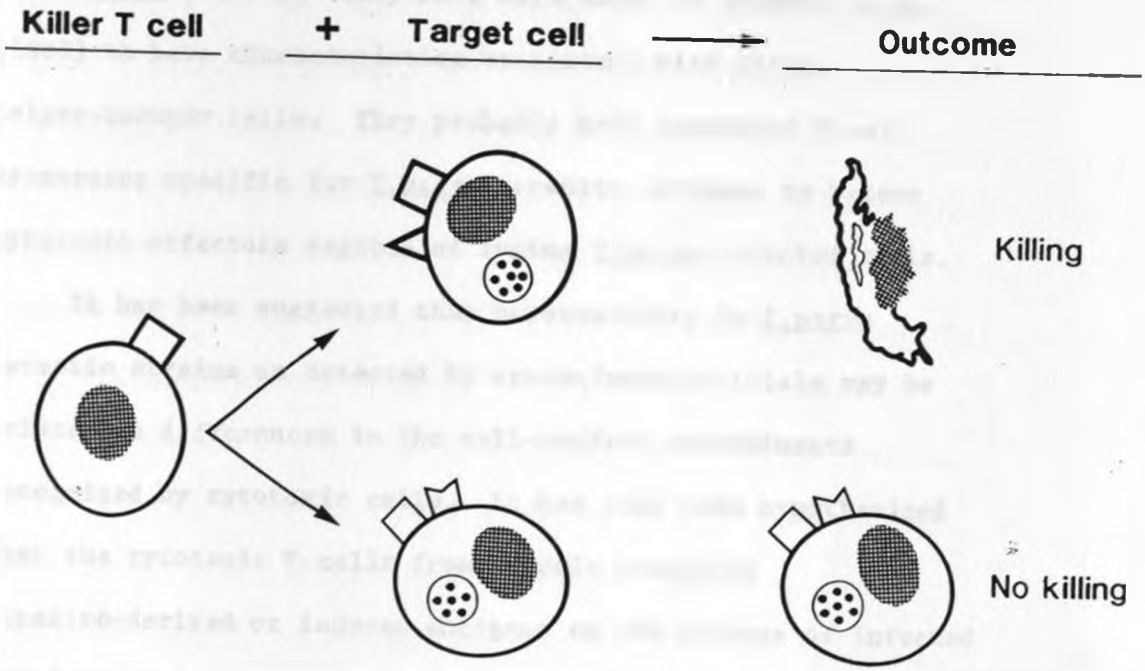


Fig. 3 Antigenic specificity of cytotoxic T cells showing how killer T cells recognize some parasite induced antigens (open triangle) on the surface of T.parva-infected cells, in conjunction with self MHC antigens (open square) but not other parasite-induced antigens (broken square) (Reproduced by kind permission of Dr. W. Ivan Morrison, ILRAD, Nairobi, Kenya).

Antigenic specificity of Cytotoxic T cells



antigens (Anon, 1982). BoT8$^+$ cytotoxic T-cells from immune cattle will not kill infected cells mismatched for both A locus determinants, that is, the killing is genetically restricted (Morrison et al., 1987a).

A second subpopulation of T-lymphocytes occur which bear the surface marker BoT4 identified by the MAb IL-A11 and IL-A12 in cattle. These cells are restricted by class II MHC antigens and are generally non-cytotoxic (Baldwin et al. 1986a, 1987; Teale et al., 1986). They have been shown by Baldwin et al. (1987) to have characteristics consistent with being helper-inducer cells. They probably help cytotoxic T-cell precursors specific for T.parva parasite antigens to become cytotoxic effectors capable of lysing T.parva-infected cells.

It has been suggested that heterogeneity in T.parva parasite strains as detected by cross-immunity trials may be related to differences in the cell-surface determinants recognized by cytotoxic cells. It has also been hypothesized that the cytotoxic T-cells from buffalo recognize parasite-derived or induced antigens on the surface of infected cells which are common among cells infected with different parasite strains, (Baldwin et al., 1988), and in this way afford protection against a wide range of parasite strains. This could be a result of the immune system of buffalo preferentially reacting to common antigens or it could be due to a prevalence of common antigens on cells infected with stocks of T.parva parasites derived from buffalo. This recognition of common antigens (perhaps in addition to other

protective mechanisms) may contribute to the resistance of buffalo to fatal infections by T.parva parasites by rendering protection against a wide variety of strains. In immune cattle, susceptibility to infection by strains of T.parva parasites which were not used for the immunization could be due to an inability to recognize the majority of common antigens, or it may be due to a low-level expression of common antigens or lack thereof on cells infected with most cattle-derived parasite strains. Alternatively common antigens may be heavily masked by the specific antigens. For instance cattle immunized with T.parva (Muguga) are susceptible to infection with T.parva (Marikebuni). But animals immunized with T.parva (Marikebuni) are protected against challenge by the immunizing strain and other strains such as T.parva (Muguga). In the former case the cytotoxic response may be weakly directed towards common antigens, totally lacking or perhaps only directed at specific antigens. Protection would therefore be limited. In contrast, immunization with T.parva (Marikebuni) may result in a potent immune response to an antigen which is expressed by cells infected with either T.parva (Muguga) or T.parva (Marikebuni).

1.7 OBJECTIVES AND EXPERIMENTAL DESIGN

The aim of this study was to evaluate the parasite strain specificity of cytotoxic cells generated in cattle immunized with 1 of 5 different stocks of buffalo-derived T.p.lawrencei

parasites or with the cattle-derived T.p.parva (Uganda) or T.p.bovis (Boleni) parasites. The objective was to determine whether any of these stocks is able to generate non-strain restricted immune T-cells, that can recognize and subsequently lyse cells infected with a wide variety of buffalo- and cattle-derived T.parva parasites. The immune T cells would therefore be recognizing parasite antigens common among T.parva parasite strains.

To evaluate the parasite specificity of immune T-cells, a group of 7 embryo transfer cattle sharing the same parents and matched for at least one BoLA A-locus specificity were immunized by infection and treatment. Cells to be used as target cells were infected with 5 stocks of buffalo-derived parasites as well as with the cattle-derived parasite stocks T.p.parva (Uganda), (Muguga), (Marikebuni), (Mariakani) and T.p.bovis (Boleni). Peripheral blood lymphocytes from the cattle which were immunized in July 1987 have been evaluated in cytotoxicity assays following stimulation in vitro with MHC-matched cells infected with the same parasite stock as was used for immunization of the animal. Lymphocytes generated in bulk cultures stimulated 1 to 4 times in vitro or generated by stimulating in limiting dilution assays (LDA) were tested for parasite strain specificity in cytotoxicity assays using as target cells the panel of cell lines infected with different parasite stocks and uninfected lymphoblasts as controls. Lymphocytes from one animal which had cytolytic activity against a large number of the cell lines, were cloned

and further tested against the entire panel of target cells in order to determine if individual immune T-cells were responsible for the broad killing through recognition of antigenic epitopes common among cells infected with various T.parva stocks. Such cells would be useful in further studies to identify common antigen epitopes.

CHAPTER 2. MATERIALS AND METHODS

2.1 IMMUNIZATION OF CATTLE WITH THEILERIA PARVA PARASITE STOCKS

2.1.1 EXPERIMENTAL ANIMALS

Seven Boran cattle (Bos indicus) of the same age (18 months), females or castrated males, were used for immunization in this study. They were derived by embryo transfer (Jordt et al., 1986) from a bull and cow which had BoLA class I phenotypes w2/w6 and KN8/KN12, repectively. The cattle identified as D487, D503 and D504 had the serologically defined BoLA class I phenotype w6/KN12, and the cattle D482, D494, D505 and D508 had the phenotype w6/KN8. All the animals were therefore matched for at least one BoLA A-locus MHC determinant. The animals were kept at the International Laboratory for Research on Animal Diseases (ILRAD) farm , Kabete, Kenya, where they were maintained indoors on hay and pelleted concentrate ration and were sprayed weekly with the acaricide dioxathion (Delnav; Wellcome Kenya, Nairobi, Kenya). They were used in this study when aged between 18 and 34 months.

2.1.2 PARASITE STOCKS AND INFECTION OF TICKS

Ticks were fed on infected cattle or buffalo in order to infect them with T.parva parasites. Three of the buffalo used for parasite stock isolation (6998, 7014, and 7065) were from the Mara and 2 (6999 and 7013) from Nanyuki. They are

maintained in captivity at the Veterinary Research Laboratories, Kabete, Kenya. The T.parva parasite stocks derived from the buffalo are referred to as T.p.lawrencei (Tpl) and designated with the appropriate buffalo number. The cattle-derived parasite stocks used are designated T.p.parva (Tpp) (Uganda) and Tpb (Boleni) (S. Morzaria, pers. comm.). The parasite stock Tpl(7014) used is ILRAD reference stock no. 3081, Tpp (Uganda) is no.3066A and Tpb (Boleni) is no. 3039A.

Rhipicephalus appendiculatus were maintained as a colony at ILRAD, and were infected as nymphs by feeding on the infected buffalo or on cattle. The nymphs fed to engorgement, dropped off and were allowed to moult and harden. They were then fed on rabbits for 4-5 days to enable maturation of the parasites in the salivary glands of the ticks into sporozoites. The sporozoite stabilates for in vivo infection of cattle and of lymphocytes in vitro were made by grinding the ticks or by dissecting their salivary glands. Salivary glands were obtained from ticks aseptically by treating them with aqueous benzalkonium chloride for 5 min then with 70% ethanol for 5 min and finally washing 3 times with sterile distilled water (Kurti et al., 1980). The glands were asceptically removed using the technique described by Mugera and Munyua (1973). The glands were homogenized in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland, U.K.) containing 10% heat-inactivated foetal calf serum (FCS) (Gibco, Uxbridge, U.K.), 2mM glutamine (Gibco), 50ug/ml gentamycin (Flow laboratories) and $5x10^{-5}$M 2-mercaptoethanol (2ME) (Merck, Darmstadt, West Germany) (complete culture medium) (20

glands/0.1ml of medium) using a micro-tissue grinder (Wheaton Scientific, Millville, NJ, USA). These were then used fresh or frozen, as required (Dobbelaere et al., 1985).

2.1.3 IMMUNIZATION OF CATTLE

The seven cattle were negative for antibodies to T.parva, Babesia bigemina, Trypanosoma vivax and Anaplasma marginale species and were clinically healthy when used. They were immunized with T.parva parasites by inoculation of 1ml of stabilate below and in front of the left ear. Five of the animals received buffalo-derived and 2 cattle-derived T.parva parasite stocks. The immunizing material used was frozen ground up adult ticks (Cunningham et al., 1973). Animals D482, D487 and D503 received ILRAD frozen stabilate no. 3081, 3039A and 3066A respectively. The other 4 cattle received frozen stabilates which do not have an ILRAD reference number. The frozen stabilates which been stored in liquid nitrogen at -140^{o}C were thawed rapidly at 37^{o} in a water bath, allowed to equilibrate for 30mins then used. Immunization involved subcutaneous inoculation of the infective tick stabilate in front of each of the animals' left ears and a simultaneous injection of long-acting oxytetracycline (TCMN-LA) (Terramycin LA; Pfizer U.K.) intramascularly. The animals were treated again after 4 days with the same dose of TCMN-LA. The Tpb (Boleni)-immunized animal D487 did not receive a second TCMN-LA dose because this stock only produced a mild infection. Tp1 (7014) was administered as 0.5 ml because of its proven

virulence (T.T. Dolan, pers. comm.). The other buffalo parasites were untested so a dose of 1 ml of the stock was administered. Cattle showing severe reactions were treated with parvaquone (Clexon; Wellcome) at a dose of 10mg/kg body weight (Dolan et al., 1984) intramuscularly. A summary of the responces to infection is presented in Table 1.

The clinical responses of cattle were monitored as follows. Rectal temperatures were recorded daily. A temperature of 39.5^{o}C or above was considered a febrile response. On the fifth day after inoculation biopsy smears were taken from the left parotid lymph node, air-dried, fixed in methanol, stained with Giemsa stain and examined for theilerial schizonts. Once schizonts were detected, peripheral blood smears and biopsy smears from the contralateral prescapular lymph node were prepared daily and stained and examined as above. The prepatent period was defined as the time in days between inoculation and the first detection of schizonts. A reaction was classified as mild when schizonts were detectable in lymph nodes for only a few days and when a febrile response was either absent or transient. A reaction was classified as severe when a marked febrile response was observed and schizonts were present in large numbers in the peripheral lymph nodes (Radley et al., 1975c; Irvin et al., 1983). Serum was collected weekly and antibody levels determined by IFA using in vitro cultured T.p.parva (Muguga) schizont antigens (Burridge and Kimber, 1972; Godeeris et al., 1982).

TABLE 1 Immunization of cattle with buffalo-derived or cattle-derived *T.parva* parasite stocks[a]

Animal No.	Parasite stock used for immunization	How stabilate stored[b]	Source of stabilate	TCMN-LA[c] dose (mg/kg)	Stabilate[d] dose	Clexon Treatment (yes or no)
D482	Tpl (7014)	frozen	Buffalo no. 7014	20	0.5 ml	No
D487	Tpb (Boleni)	frozen	Cattle stabilate	23	1 ml	No
D494	Tpl (6999)	fresh	Buffalo no. 6999	25	1 ml	No
D503	Tpp (Uganda)	frozen	Cattle stabilate	25	1 ml	No
D504	Tpl (6998)	fresh	Buffalo no. 6998	22	1 ml	Yes
D505	Tpl (7013)	fresh	Buffalo no. 7013	25	1 ml	No
D508	Tpl (7065)	fresh	Buffalo no. 7065	22	1 ml	No

a All the cattle except the Tpb (Boleni)-immunized animal were given a second dose of TCMN-LA at 7 days post-inoculation (same dose per animal as outlined above).

b Fresh or frozen ground up infected tick material was used.

c TCMN-LA, tetramycin injectable solution (Pfizer, UK).

d Stabilate dose, given on the left ear.

2.2 ESTABLISHMENT OF THEILERIA PARVA-INFECTED CELL LINES

2.2.1 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Blood was collected from the jugular vein with a 16-gauge needle into a syringe containing an equal volume of Alsevers solution (Emery et al., 1981; Lalor et al., 1986). All further operations were performed aseptically in a horizontal laminar air flow hood (Flow Laboratories). Aliquots of 30 ml anti-coagulant treated blood were layered onto 20 ml ficoll/diatrizoate sodium solution of specific gravity 1.077 (Ficoll-paque, Pharmacia Fine Chemicals, Uppsala, Sweden)) in 50 ml polypropylene conical tubes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ. U.S.A.) and centrifuged at 900xg for 30 min at 23^{o}C. PBM were aspirated by pipette from the interphase with the red blood cells, diluted with an equal volume of Alsevers and pelleted by centrifugation (10 min at 450xg, 23^{o}C). The PBM were washed 3 times in Alsevers solution by repeated suspension and centrifugation (10 min at 180xg, 23^{o}C) to remove platelets. The cell population was then suspended in complete medium. The batch of FCS was selected by screening for support of cell proliferation in mixed leucocyte reactions (MLR). The PBM preparation was used for establishment of T.parva infected cells, generation of cytotoxic cells and limiting dilution assays.

2.2.2 SPOROZOITE STOCKS FOR INFECTION OF LYMPOCYTES

The sporozoite stocks used for the infection of lympocytes were frozen stabilates of dissected tick salivary glands (section 2.1.2). The stocks from buffalo no. 6998, 6999, 7013 and 7065 do not have ILRAD reference numbers. The remaining stocks used were Tpl (7014), (Chumo *et al*. 1985); Tpb (Boleni), (Lawrence and Mackenzie, 1980); Tpp (Muguga), (Brocklesby *et al*.,1961); Tpp (Mariakani), (Irvin *et al*., 1983); Tpp (Marikebuni), (Irvin *et al*., 1983) and Tpp (Uganda) (Lawrence and mackenzie, 1980).

2.2.3 INFECTION OF LYMPHOCYTES WITH SPOROZOITES

Two of the 7 experimental animals, D482 and D504, served as donors of PBM for *in vitro* infection with *T.parva* sporozoites. The PBM were isolated from the blood collected from the 2 animals before they were immunized. The infection was established by the additon of aseptically derived salivary glands containing sporozoites (as described in section 2.1.2) to the PBM as described previously (Brown *et al*., 1973; Brown, 1979; Baldwin *et al*., 1986b)

PBM were counted and concentrated to 1-5 x 10^6 cells/ml in 0.5-1 ml culture medium and 0.2 ml of salivary gland homogenate added in polycarbonate tubes. The concentrate was then mixed on a vortex mixer and incubated for 2 hr at 37^{o}C, shaking every 20 min. Finally 10 ml of fresh medium was added and the tube centrifuged (180xg, 10 min). The pelleted cells were resuspended in fresh complete culture medium and

transferred to 25 cm^2 tissue culture flasks and incubated at 37°C in a humidified incubator (Heraeus Christ, Osterode, West Germany) in an atmosphere of 5% CO_2 in air. The established cell lines were used as stimulator cells in proliferation assays, for generation of cytotoxic cells and as target cells in in vitro cytotoxicity assays.

2.2.4 MONOCLONAL ANTIBODY TYPING OF PARASITES

Cell lines infected with buffalo or cattle-derived parasite stocks, were tested with monoclonal antibodies (MAb) to Theileria schizont antigens in the immunofluorescence antibody assay (IFA) inorder to compare their antigenic composition (Irvin et al., 1983; Conrad et al., 1987b). MAb 1-7 were raised against T.p.parva (Muguga)-infected cells by Pinder and Hewett (1980). MAb 8 to 13 were raised against T.p.parva (Kiambu 5)-infected cells and MAb 14 to 16 against T.p.parva (Marikebuni)-infected cells (Minami et al., 1983). MAb 17-20 were raised against T.p.lawrencei schizonts (Newson et al., 1986).

Cells for schizont antigen testing were prepared from in vitro cultivated T.parva-infected cells in the log phase of growth. Culture medium containing approximately 2 x 10^8 cells was centrifuged at 200xg for 15 min at 4°C. The pellet was resuspended in 0.01M phosphate buffered saline (PBS) (pH 7.2) maintained at 4°C and the suspension passed through a Ficoll-paque gradient solution by centrifuging at 700xg for 20 min at 4°C. The separated layer of viable cells was obtained

from the Ficoll-paque interface and resuspended in 10 ml of PBS. The cells were washed 3 times in PBS (200xg for 10 min at 4°C). Finally the cells were resuspended in 4 ml of PBS and cell counts determined. An equal volume of fixative (3.7 per cent formaldehyde buffered in PBS, pH 7.2) was added drop by drop with gentle stirring at 4°C. Fixation was completed by allowing the cell suspension and fixative to roll on a roller mixer (coulter) for 15 min at room temperature. After fixing, the cells were washed 3 times (200xg for 10 min) and finally resuspended in 2 ml of PBS buffer. The cells were counted and adjusted to 1-2 x 10^7 cells/ml in PBS. They were then distributed onto Wellcome PTFE multispot slides (C.A. Handley, Essex, UK) by adding one drop to each well and immediately sucking off the liquid with a micropipette. In this way approximately 40 x 10 well slides could be prepared from each 1 ml of fixed cell suspension (Minami et al., 1983). Slides were air dried at room temperature, wrapped in aluminium foil and stored at -20°C or -80°C.

The IFA test used was based on the procedure described by Goddeeris et al. (1982) for the detection of T.parva schizont antigens and their characterization using MAb. The antigen slides were thawed by placing onto immunoframes and leaving for 15-20 min at room temperature. Monoclonal antibodies were diluted in cold (4°C) PBS (pH 7.2) to a baseline of 1:50. Further dilutions of 1:200, 1:800 to 1:51,200 were made, and 20 ul of each dilution was added to a well of the test slides. The slides were incubated in a moist chamber for 30 min at room

temperature. They were then washed with cold PBS 3 times (each wash was for 10 min in fresh PBS) without drying. 20 ul of rabbit anti-mouse IgG conjugated with flourescein isothiocyanite (Miles laboratories, Slough, UK) containing 0.01% Evans blue was added to each well. The slides were incubated in a moist chamber for 30 min. Excess conjugate was washed off the slides with PBS 3 times as described above, and immediately mounted in 50% glycerol in PBS (pH 8.0). Slides were examined for flourescence using an orthoplan flourescence microscope (Ernst Leitz, Wetzlar, West Germany). A positive reaction appeared as a green flourescing schizont.

2.2.5 DERIVATION OF CLONED PARASITIZED CELL LINES IN VITRO

PBM from animal D482, which had been infected *in vitro* with sporozoites of Tpl (7014) and (7013) (section 2.2.3) and cultured in complete culture medium (section 2.2.1) for 7 days, were cloned by limiting dilution. The level of parasitosis in the culture was about 60% at the time of cloning. The cloning procedure was done by distributing the cells into 96-well round-bottom microtitre plates (Flow laboratories) in complete culture medium supplement with 10% T-cell growth factor (TCGF). The TCGF was conditioned medium from PBM stimulated with concanavalin A (Con A) (type iv; Sigma, St. Louis, MO, U.S.A.) (Teale *et al.*, 1985). The cells were seeded at 10, 3, 1 or 0.3 cells/well and half of the culture medium was replaced every 3-4 days. After 10 days the wells were examined for cell growth and cultures growing at seeding densities which gave

less than 35% wells with cell growth were subsequently transferred for expansion, first into 48-well culture plates (Costar, Cambridge, MA, U.S.A) and subsequently into 25 cm^2 tissue culture flasks. The majority of these cultures were considered to have arisen from infection of a single cell. TCGF was withdrawn from the cultures when they were transferred to 48-well culture plates. These clones were used as stimulator cell lines for generation of cytotoxic T lymphocytes from PBM of immune cattle and as target cell lines in *in vitro* cytotoxicity assays.

2.2.6 MAINTENANCE AND STORAGE OF CELL LINES

The infection of lymphocytes with a particular buffalo- or cattle-derived parasite stock was confirmed using anti-*T. parva* schizont MAb in the IFA (section 2.2.4). In addition cytospin smears were made by diluting cells to 1 x 10^5/ml in about 0.3 ml of medium and centrifuged at 60xg (Cytospin; Shandon Southern Instruments, Sewickley, PA, USA) onto glass microscope slides. The spread was air dried, fixed in absolute methanol, hydrolyzed with 1N HCl for 5 min at 60°C, rinsed 3 times in distilled water, air dried and stained with Giemsa (6% in 5 x 10^{-4}M NH_4OH) at 37°C for 30 min (Kurti *et al.*, 1980). The slides were then examined under the microscope.

Cells were maintained by routine subculture while in the exponential phase of growth by diluting in medium. Complete culture medium (see section 2.1.2) was used at all times, and the cultures were expanded as required.

Once established, frozen stabilates of all the cell lines were made. Cells were centrifuged (180xg, 10 min) and the pellet suspended in FCS at a maximum of 6×10^7 cells/ml. Dimethyl sulphoxide (DMSO) (Merck) was added drop by drop while cooling the tube on ice. One part DMSO was mixed with 3 parts FCS. The tubes were transfered into plastic screw-cap vials (Nunc, Roskilde, Norway) for liquid nitrogen storage. The cells were cooled evenly from the time of adding the DMSO, without allowing the cells to warm again. The vials were then rapidly transfered to insulated hollow jackets suspended in the neck of a liquid nitrogen cylinder (Kelvinator, Manitowoc, WI, U.S.A.) and left overnight in the vapour phase of the liquid nitrogen cylinder. After 24 hr the cells were transferred into the liquid phase of the nitrogen for long-term storage.

The cells were resuscitated in a water bath at $37^{\circ}C$. The cell suspensions were then transferred from the vials to sterile tubes and about 3 ml of warm FCS added dropwise. The cells were spun into a pellet and washed once or twice to remove DMSO. It is important to rapidly reduce the concentration of DMSO around the cells when they are warmed up as the DMSO may inhibit growth and proliferation. The cells were then counted and cultured as described above.

2.2.7 BOLA TYPING OF INFECTED CELL LINES

PBM and parasitized cell lines were typed for their BoLA phenotype using alloantisera and MAb in a microlymphocytotoxicity assay (Teale *et al.*, 1983). The

majority of the reagent define class I MHC specificities (Spooner et al., 1979) encoded by a single locus, the BoLA-A locus (Anon 1982). The specificities w6, w7 and w10 are internationally defined while KN8 and KN104 are locally defined (Teale et al., 1985).

2.3 ASSESSMENT OF CYTOTOXIC CELLS FROM THE BLOOD OF IMMUNE CATTLE IN RESTIMULATED BULK CULTURES.

2.3.1 AUTOLOGOUS THEILERIA MIXED LEUCOCYTE REACTION

Peripheral blood mononuclear cells were obtained from blood collected into Alsevers solution from the 7 immunized animals (Section 2.2.1) or from blood collected into a conical flask containing glass beads (5 mm diameter) which was defibrinated by gently swirling the blood and beads for 10 min (Goddeeris et al., 1985). The PBM were isolated as described above (section 2.2.1) suspended in culture medium and assayed in 96-well flat-bottom microtitre plates (Costar) for proliferative responses to T.parva-infected cells in MLR (Goddeeris and Morrison, 1987). 5×10^5 PBM were added per well in a final volume of 200 ul per well. The stimulator cells were lymphocytes infected with 1 of the 7 T.parva parasite strains used for immunization of the cattle. The number of stimulator cells ranged from $1x10^6$/well to $1x10^3$/well. The stimulator cells were exposed to 5000 rads of gamma-radiation from a ^{137}caesium source prior to use. Cultures were incubated at 37°C in a humidified atmosphere of

5% CO_2 in air. The optimal time of incubation was 5 days for Alsevers PBM (which contained 5-20% monocytes) or 6 days for PBM from defibrinated blood which was depleted of monocytes (Goddeeris and Morrison, 1987). All cultures were set up in triplicate.

Proliferation of cells was measured by the incorporation of [^{125}I] iododeoxyuridine (IUDR), 0.5 uCi/well over 8 hr (Amersham International, Aylesbury, Buckinghamshire, England, U.K.) by multiplying cells (Lalor et al., 1986). Cells were harvested with a cell harvester (Dynatech, Zug, Switzerland) onto glass fibre filters (Flow laboratories) and radioactivity counted using a gamma counter (Beckman, Geneva, Switzerland). Results were expressed as counts per minute (CPM) of radioactivity per well (Lalor et al., 1986).

As a control PBM was also assayed for responses to the lectins Con A (Sigma) at 2 ug/ml, and phytohaemagglutinin (PHA-P; Difco, Detroit, MI, U.S.A) at 10 ug/ml, final concentration in the well. Preliminary experiments using 2.5 x 10^5 PBM/well had shown that these lectin concentrations induced optimal responses of Alsevers PBM (Goddeeris et al., 1985) when PBM were compared over a range of cell concentrations (Baldwin et al., 1985). The final volume in the well was 200 ul. Cultures were incubated in an atmosphere of 5% CO_2 in air and proliferation was assayed after 72 hr by incorporation of IUDR counted and results expressed as described above.

2.3.2 ESTABLISHMENT OF UNINFECTED LYMPHOCYTE BLASTS

Uninfected lymphoblasts for use as control target cells in cytotoxicity assays were obtained by stimulating PBM with the lectin Con A (2.5 ug/ml) and subsequently maintained as cell lines by subculture in complete medium with 15% bovine TCGF every 3-4 days. One day before use as targets 100 mM alpha-methyl mannoside (Sigma) was added to the culture medium to block binding sites of any residual Con A present in the TCGF supernatant (Baldwin *et al.*, 1988).

2.3.3 GENERATION OF CYTOTOXIC CELLS IN BULK CULTURES

All 7 experimental animals served as donors of PBM for *in vitro* generation of cytotoxic cells which were then used as effectors in cytotoxicity assays. The cytotoxic cells were assessed for parasite specificity and MHC restriction after a series of weekly restimulations. Cultures for generation of cytotoxic cells were initiated from the experimental animals at least 2 months after immunization.

PBM were obtained from venous blood collected into Alsevers solution or defibrinated as described above (section 2.2.1 and 2.3). Gamma-irradiated (5000 Rads) *T.parva*-infected stimulator cells were added to the wells of 24-well culture plates (Costar) in a total volume of 2 ml culture medium without HEPES buffer. The irradiated parasitized cells (stimulators) are non-proliferative but the PBM (responders) are able to proliferate in response to antigens on the surface of the irradiated parasitized cells. The stimulator cell

concentration used varied with the different stimulator cell lines, but the concentration used was that which gave optimal proliferation in a unindirectional MLR (section 2.3.1). After 7 days cells were collected from the wells, and viable cells were separated on Ficoll-paque gradients (900xg for 30 min at 23°C), washed once with RPMI 1640 medium (180xg for 10 min at 23°C) and stimulated for a second time with irradiated parasitized cells as in the primary culture. After 1 week, viable cells were separated as before and stimulated for a third time at 2×10^6 cells/well with 1×10^6 or 3×10^5 gamma-irradiated autologous parasitized cells. At this time, cultures were supplemented with 2×10^6 gamma-irradiated (5000 rads) autologous PBM as filler cells. Experiments by Goddeeris et al. (1986a and b) indicated that filler cells were needed in restimulated bulk cultures to maintain good proliferation. Viable cells recovered following the third stimulation were further restimulated at weekly intervals in aliquots of 1×10^6 cells/ml with 1×10^6 or 3×10^5 irradiated infected cells and 3×10^6 autologous irradiated PBM cells as fillers. Cultures were terminated after the fourth stimulation. The cultured cells were tested for cytotoxic activity after 2, 3 and 4 stimulations.

2.3.4 CYTOTOXICITY ASSAYS

Cytotoxic activity of viable lymphocytes (effectors) recovered after each in vitro stimulation in the autologous Theileria mixed leucocyte culture (MLC) was measured in a 4

hr ^{111}indium (^{111}In)-release assay (Goddeeris et al., 1986b). The lymphocytes were tested for their cytolytic activity using as target cells:

a) MHC-matched lymphocytes (either from D482 or D504) infected with various buffalo-derived stocks of T.parva or infected with cattle-derived parasites T.p.parva (Muguga), T.p.bovis (Boleni), T.p.parva (Uganda), T.p.parva (Marikebuni) and T.p.parva (Mariakani).

b) Mitogen-stimulated autologous uninfected lymphoblasts as control targets (section 2.3.2).

The target cells were established and maintained in culture as described previously (Section 2.2.3 and 2.2.6). On the test day, the target cells were obtained from cultures in exponential growth phase and suspended in cytotoxicity medium. Cytotoxicity medium consisted of RPMI-1640 medium, 20 mM HEPES buffer, and 10% FCS. The batch of FCS is selected so as to give low spontaneous release of the radioactive label from target cells. The cells were labelled with ^{111}In oxine (Amersham International). Aliquots of 50 ul containing $1x10^6$ cells were incubated with 5 uCi (1ci = 37 GBq; 1Bq = 1 disintegration per second) of ^{111}In oxine at 39^{o}C for 15-20 min. Following incubation, the labelled cells were pelleted by centrifugation (10 min, 300xg) and washed 6 times in 10 ml of cytotoxicity medium and finally resuspended in 10 ml of medium.

Effector lymphocytes were collected from 24-well culture plates (Costar) and transfered into polycarbonate tubes (Falcon) and centrifuged (10 min, 300xg) and a viable count

obtained by Trypan blue exclusion. The effectors are added to target cells over a range of effector to target (E:T) ratios. To start at an E:T ratio of 40:1, the effector cells are adjusted to 2 x 10^6/ml. 100ul are added in duplicate to V-bottom wells of 96-well microtitre plates (Greiner, Wurtingen, West Germany). The remainder of effectors are diluted 1:2 to make subsequent E:T ratio of 20:1 and the procedure repeated until the final desired E:T ratio is obtained. 50 ul of 5 x 10^3 ^{111}In-labelled target cells were then added to the wells. Cells in the wells were pelleted by centrifugation (7 min, 160xg) and incubated for 4 hr at 39^{o}C in a humidified atmosphere of 5% CO_2 in air.

To prepare medium controls which determine the spontaneous release of the radioactive label, 100 ul of cytotoxicity medium is mixed with 50 ul of ^{111}In-labelled target cells for each target cell line tested.

To prepare freeze-thaw controls, which give the maximum possible release of radioactive label, 50 ul of ^{111}In-labelled target cells is added to 100 ul of distilled water in Eppendorf tubes (Eppendorf, Hamburg, West Germany) and subjected to 2 cycles of quick freeze in liquid nitrogen followed by slow thawing at room temperature.

Following incubation of the target and effector cells, the cells are resuspended, centrifuged (5 min, 180xg), 75 ul of supernatant harvested from the wells and radioactivity counted using a Gamma Counter. Percentage cytotoxicity was calculated as:

$$\% \text{ cytotoxicity} = \frac{\text{(test release - spontaneous release)}}{\text{(maximum release - spontaneous release)}} \times 100\%$$

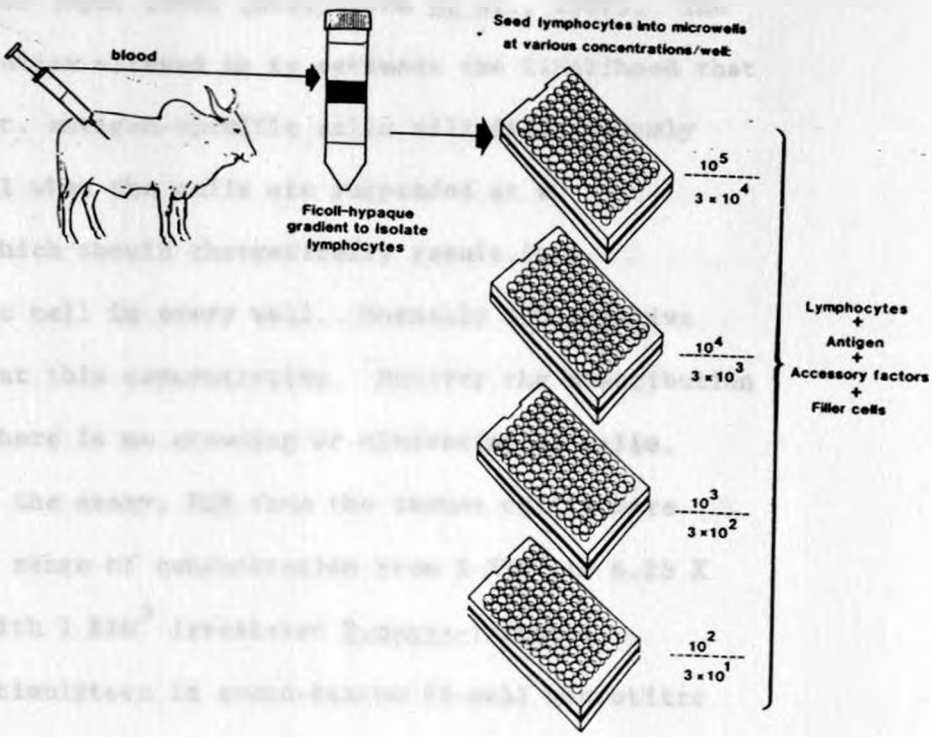
2.4 LIMITING DILUTION ASSAYS

2.4.1 GENERATION AND ASSAY OF CYTOLYTIC T-CELL PRECURSORS

Limiting dilution assays were carried out to determine the precursor frequencies of cytotoxic T-cells with various parasite specificities in the blood of the cattle immunized with the different parasite stocks. The principle of the system is based on activating the precursors of antigen-specific cytotoxic lymphocytes within the PBM population by 1 antigen stimulation *in vitro* (Waldmann and Lefkovits, 1984). T-cells of a single antigen specificity occur in very low frequencies in lymphocyte populations, about 10^{-4} to 10^{-7} in suspensions from unprimed animals and frequencies 10 to 100-fold higher in suspensions from primed or immune animals (Henry *et al.*, 1980). The LDA determines the fraction of cultures (i.e. wells with PBM) that are negative for the desired effector responce over the range of cell concentrations tested and does not attempt to determine how many antigen-specific cells are in the positive wells. If the assay is properly designed, a fraction of negative cultures is observed for several cell concentrations tested. To achieve this, decreasing numbers of cells of the sample under consideration are cultured to cover a range of dilutions in which only a fraction of wells (cultures) have cells which display the responce being tested for (Fig. 4). When cytotoxicity is the effector responce being assayed for, normally cells in a single well which yields more than 10%

Fig. 4 Limiting dilution analysis for estimation of the frequency of precursor cytotoxic T cells in the PBM of immunized cattle. The PBM was separated from blood by gradient centrifugation and seeded into microwells at various concentrations and stimulated with gamma-irradiated T.parva-infected cells and 15% TCGF. They were incubated for 7 days in 96-well round bottom microtitre plates and assayed for parasite specificity by an ^{111}In-release cytotoxicity assay. Precursor frequencies were determined by the maximum likelihood estimation described by De st. Groth (1982). (Reproduced by kind permision of Dr. C.L. Baldwin, ILRAD, Nairobi, Kenya).

Limiting Dilution Analysis



specific lysis is scored as positive (Henry et al., 1980). This chosen arbitrary percentage should be a figure well above any lysis due to errors in isotope counting or experimental manipulations, so that the calculation of the total number of positive wells (cultures) is a conservative estimate. There should be a linear relationship between the logarithm of the fraction of negative wells and the number of T-cells cultured at each responder input level (Strijbosch et al., 1987). The Poisson distribution allowed us to estimate the likelihood that 0, 1, 2 or 3 etc. antigen-specific cells will fall randomly into a microwell when the cells are suspended at a concentration which should theoretically result in 1 antigen-specific cell in every well. Normally 37% negative cultures occur at this concentration. However the distribution holds only if there is no crowding or clustering of cells.

To conduct the assay, PBM from the immune cattle were cultured over a range of concentration from 2 $X10^4$ to 6.25 X 10^2cells/well with 1 $X10^3$ irradiated T.parva-infected cells/well as stimulators in round-bottom 96-well microtitre plates (Flow Laboratories). The stimulator cells were infected with the Theileria parasite stock which was used to immunize the animal from which the PBM were obtained. Thirty-six microwells were established for each responder cell concentration. A seventh replicate group of stimulators plus medium alone was set up as a control group. TCGF was added to a final concentration of 2.5%/well. Cultures were incubated for 7 days at 37^0C in a humidified atmosphere of 5% CO_2 in

air at the end of which individual wells were tested for cytotoxic activity in a 4 hr ^{111}In-release assay. The target cell lines included the autologous T. parva-infected cell line used for stimulation as well as cell lines infected with other buffalo or cattle-derived T.parva parasites. Each individual well was split (to assay two target cell lines) and transferred to V-bottom plates (Greiner). The rest of the assay proceeded as described above (section 2.3.4).

2.4.2 ANALYSIS OF LIMITING DILUTION ASSAY RESULTS

The analyses of results from the ^{111}In-release assay are straight forward. A well is taken to be negative if its value is less than the mean obtained from the control wells plus 3 standard deviations (Buurman et al., 1983). The level of cytotoxicity per well is obtained from its relation to the means of the maximum and background release. A negative value indicates the well has a count less than the mean background level. From this cytotoxicity data, the concentration of precursor cytotoxic T-lymphocytes in the PBM population is obtained from the gradient of a plot of the natural logarithm of negative cultures versus the responder cell input, with the line passing through the origin. A computer programme has been written to carry out these calculations based on the maximum likelihood estimation (De st Groth, 1982) (compliments of Dr. J.R. Young, ILRAD). The programme also calculates the 95% confidence limits, chi square (X^2) and correlation coefficient (R^2) values of this result. An R^2 value indicates how well the data fit a straight line and only data

with R^2 value of 0.7-1.0 were used. The X^2 value indicates the goodness of fit of a linear model to the data and should be less than one (only results with X^2 values less than 1 were considered valid). Table 2 gives the results for one assay and Fig. 5 is a plot of the natural logarithm of negative cultures versus the responder cell input with the line passing through the origin.

2.5 CLONING OF CYTOTOXIC T-CELLS

The cloning of cytotoxic T-cell is summarized in Fig. 6 and by Goddeeris and Morrison (1988).

2.5.1 COMPLEMENT-MEDIATED LYSIS OF BOT4$^+$ CELLS

Approximately 1ml of 1 X 10^7 viable cells from bulk cultured PBM which had been restimulated 3 times *in vitro* with gamma-irradiated T.parva-infected cells, were incubated with 0.5 ml of MAb IL-A11 (1/250 dilution in culture medium) in a 10 ml polycarbonate tube for 40 min at 4^0C. Complement solution (0.5ml; Cederlane Laboratories, Hornby, Ontario, Canada) was then added to the mixture and incubated for 40 min at 38^0C. The complement solution was made by dissolving 1 vial of lyophilized low toxicity rabbit complement in 1 ml of distilled water and 1ml of RPMI 1640 medium (without HEPES). Several batches of complement were tested for low spontaneous cytotoxicity on bovine lymphocytes and the batch with the lowest spontaneous cytotoxicity was selected for further use.

TABLE 2 Experimental data from limiting dilution assays of PBM from animal D508, Tpl (7065)-immunized, tested on target cells infected with Tpp (Marikebuni)[a]

Cells per well	No. of wells	No. of negative cultures[b]	Proportion of negative wells	Log proportion negative	Mean cytotoxicity	SD
20000	36	0	0.00	–	36.03	16.49
10000	36	0	0.00	–	31.08	17.76
5000	36	2	0.06	-2.89	29.65	14.88
2500	36	11	0.31	-1.19	12.96	7.13
1250	36	17	0.47	-0.75	11.16	9.74
625	36	31	0.86	-0.15	2.35	6.70

a The responder cells (PBM) from immune animal D508 were cultured over a range of concentrations with T.parva-infected gamma-irradiated stimulator cells for 7 days and assayed for specific cytolysis on T.p.parva (Marikebuni) infected cells. The 95% confidence limits for this result was 0.00026 and the Chi square (X^2) = 0.026: correlation coefficient (R^2) = 0.9345 (NB, A result was only taken to be valid if the X^2 value was less than one and the R^2 value close to one (0.70-1.0).

b Culture wells were scored as negative for precursor cytotoxic lymphocytes if the ^{III}In-release value was less than 3 standard deviations above the mean background level (Mean = 374.56 + 3 SD (1 SD = 83.29) = 624.41 = cut off point).

Fig. 5 Semilog plot of PBM input against the log of non-responding cultures as determined in the limiting dilution assay. PBM from immune steer D508 were stimulated *in vitro* with Tpl (7065)-infected cells over a range of PBM concentrations (X-axis). The cultures were then assessed for cytotoxicity on the stimulator cell line. In this single hit curve the frequency of cytotoxic T cells can be interpolated from f = -0.20. The small squares represent the actual data points from the assay, the big squares represent the best fit line through these data points while the star points represent an attempt to draw the best fit line through the origin. The estimated frequency of cytotoxic cells in the PBM which are able to kill the infected cell line is about 1/1580.

Log. of the natural fraction of non-responding cultures

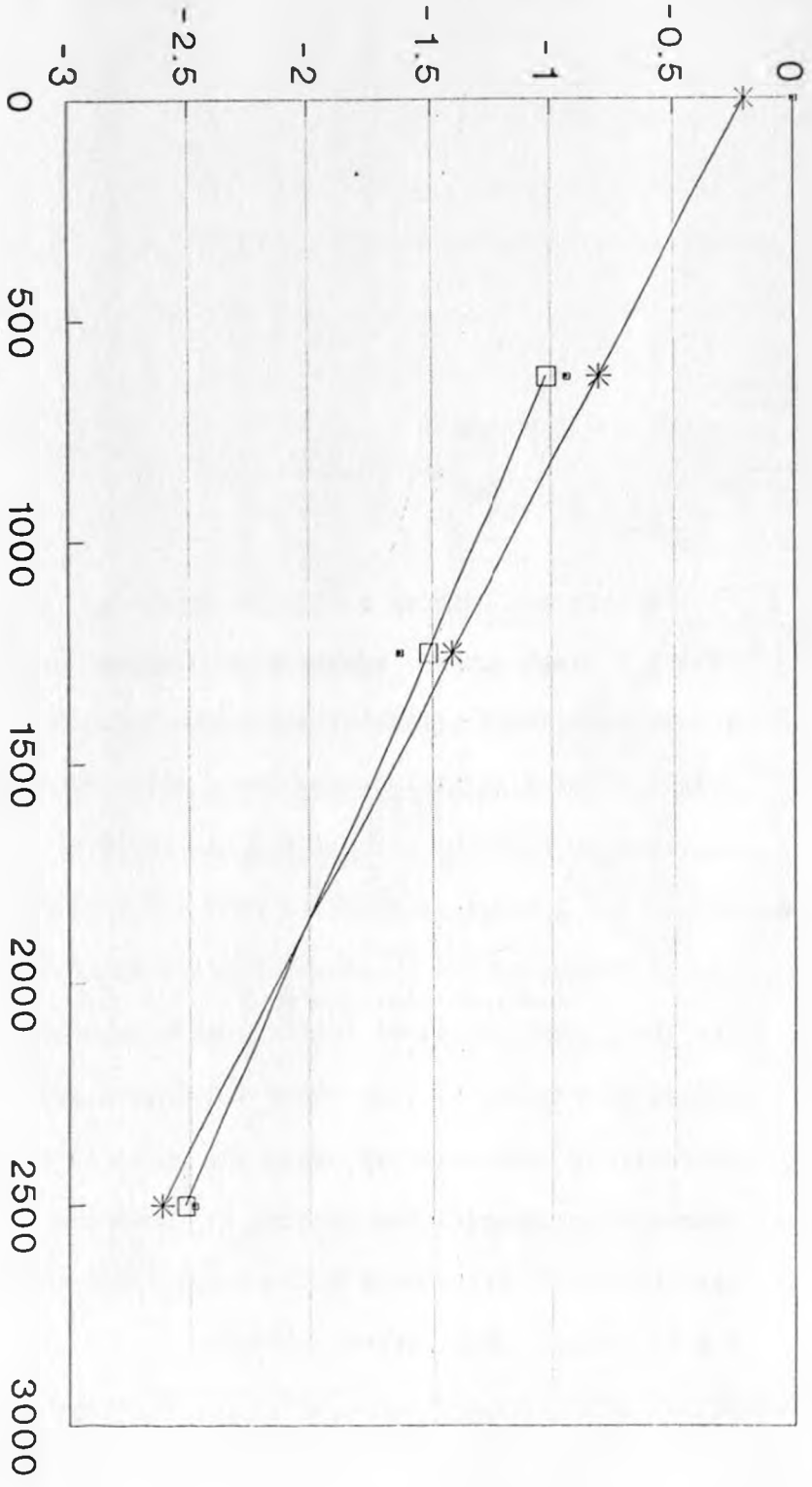
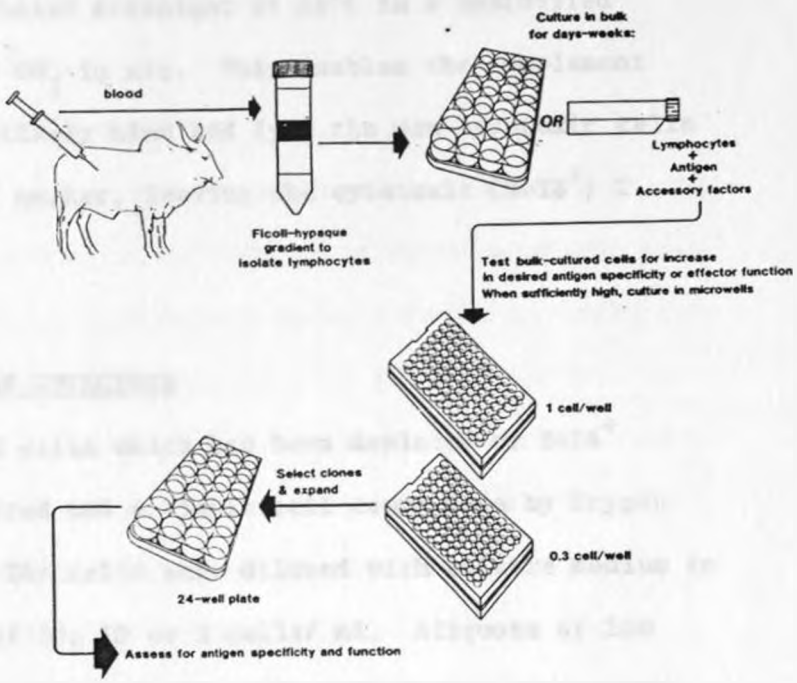


Fig. 6 Propagation and cloning of Theileria-specific bovine T lymphocytes. Lymphocytes from an immune animal containing precursor cytotoxic T cells specific for T.parva-infected cells were stimulated in vitro in bulk cultures with gamma-irradiated autologous T.parva-parasitized cells to increase the frequency of the cytotoxic T cells specific for the T.parva-infected cells. These were then cloned by seeding at 1 or fewer cells/well and stimulating with infected cells and TCGF. Selected clones were expanded for testing for parasite specificity. (Reproduced by kind permision of Dr. C.L. Baldwin, ILRAD, Nairobi, Kenya).


Culture in bulk
for days-weeks:
blood
OR
Lymphocytes
+
Antigen
+
Accessory factors
Ficoll-hypaque
gradient to
isolate lymphocytes
Test bulk-cultured cells for increase
in desired antigen specificity or effector function
When sufficiently high, culture in microwells
1 cell/well
Select clones
& expand
0.3 cell/well
24-well plate
Assess for antigen specificity and function

Some batches of complement solution were preabsorbed with bovine lymph node cells to remove spontaneous cytotoxicity (Lalor et al., 1986). After incubation the cells were diluted in 8ml of culture medium and pelleted by centrifugation (180xg, 10 min, room temperature). The pellet was resuspended in 2 ml of culture medium (without HEPES buffer) with 10% TCGF, the cells transferred into a well of a 24-well culture plate (Costar) and incubated overnight at $38^{0}C$ in a humidified incubator with 5% CO_2 in air. This enables the complement solution to selectively bind and lyse the non-cytotoxic cells bearing the $BoT4^{+}$ marker, leaving the cytotoxic ($BoT8^{+}$) T lymphocytes.

2.5.2 CLONING OF EFFECTORS

The cultured cells which had been depleted of $BoT4^{+}$ cells were harvested and a viable cell count made by Trypan blue exclusion. The cells were diluted with culture medium to a concentration of 30, 10 or 3 cells/ ml. Aliquots of 100 ul/well were distributed in 96-well round-bottom microtitre plates. 5 plates with 0.3 cells/well, 5 plates with 1 cell/well and 2 plates with 3 cells/well were made. Aliquots of 100 ul of a cell suspension containing 5×10^{4} irradiated autologous infected cells/ml, 2×10^{5} irradiated autologous PBM/ml and 30% TCGF in culture medium (without HEPES), were added to all the wells. The plates were incubated at $38^{o}C$ in a humidified incubator with 5% CO_2 in air.

2.5.3 SCREENING WELLS FOR GROWTH

After 2 weeks of culture, wells were screened for cell growth. Cells from wells which were estimated to have more than 10^3 cells were tested for cytotoxic ability in an ^{111}In-release assay using as target cells the same infected cell line used as the stimulator. Only cultures growing at seeding densities which resulted in less than 35% of wells seeded having cell growth were selected for expansion.

2.5.4 INDIUM RELEASE ASSAY

Target cells were prepared and labelled as described above (Section 2.3.4). 100 ul of each selected well containing the putative cytotoxic cell clone was transferred aseptically into a well of a 96-well V-bottom microtitre plate (Greiner), the plate centrifuged to pellet the cells (180xg, 5 min at room temperature) and the culture medium decanted from the well. The cells were immediately resuspended in 100 ul of cytotoxicity medium using an eight channel pipette (Flow Laboratories). 50 ul of radiolabelled target cell suspension containing 5 X 10^3 cells was added to each well. The rest of the procedure is as described for the ^{111}In-release assay (section 2.3.4).

2.5.5 EXPANSION OF THE CLONED CELLS

The cells selected for expansion were those which exhibited cytotoxic activity and which originated from cultures seeded at a cell concentration which gave rise to cell growth

in less than 35% of the wells, as these have a probability of greater than 83% of being clones. The remaining cells in the well of a selected culture were harvested and resuspended in 10 ml of culture medium (without HEPES). Aliquots of 100 ul were added into each well of a 96-well round-bottom microtitre plate using an Oxford microdoser (Fischer Scientific, Springfield. NJ. USA). 100 ul of a cell suspension containing 5 X 10^4 autologous irradiated infected cells/ml and 30% TCGF in culture medium (without HEPES) were then added to each well and the plates incubated at 38^0C in a humidified incubator containing 5% CO_2 in air. After 2-3 weeks cloned cells were further expanded in 96-well round-bottom microtitre plates as described above or in 24-well culture plates by seeding 2 X 10^5 cloned cells and 1-2 X 10^6 irradiated infected cells/well in a total volume of 2 ml culture medium (without HEPES buffer) containing 15% TCGF.

2.5.6 TESTING CLONED EFFECTOR CELLS FOR PARASITE SPECIFICITY

Cloned effector cells were evaluated for parasite specificity by testing a number of different infected cell lines and uninfected blasts as target cells in the ^{111}In-release assay as described above for the bulk cultured cytotoxic cells (Section 2.3.4).

CHAPTER 3. RESULTS

3.1 IMMUNIZATION OF CATTLE AND ASSESSMENT OF THEILERIA PARVA PARASITE STOCKS

3.1.1 MONOCLONAL ANTIBODY PROFILES OF SCHIZONT ANTIGENS

To compare the parasite stocks used to immunize cattle and infect cell lines, Theileria schizonts antigens were typed using MAb in the IFA. The binding of MAb to schizonts of cells infected with different T.parva stocks is summarized in Fig.7. MAb 1, 4-6, and 8-13 bound to schizonts of all the 20 infected cell lines indicating that they react with antigenic determinants common among cells infected with the various buffalo and cattle-derived T.parva parasites. Antibodies 2 and 3 only bound to T.p.parva (Muguga) infected cells indicating expression of antigenic determinants different from those expressed by other cell lines. Similarly MAb 14, 15, 16 and 18 gave variable results with the different cell lines whereas MAb 20 reacted with schizonts of all cells infected with buffalo-derived T.parva parasites but not those of cells infected with cattle-derived parasites. MAb 17 and 19 did not react with any of the cell lines and MAb 7 reacted with all but the Tpb (Boleni)-infected cells.

Within the group of cells infected with the buffalo-derived T.parva parasite stocks, the profile for cell lines D482 and D504 Tp1 (7065)-infected and D504 Tp1

Fig. 7 Monoclonal antibody profiles of schizont antigens of PBM infected with buffalo-derived parasite stocks Tpl (7014), (7013), (6998), (6999), or (7065) or with cattle-derived Tpp (Marikebuni), (Mariakani), (Muguga) or (Uganda) or Tpb (Boleni), as analysed by the IFA.

Theileria parva Parasite Stocks	Number of monoclonal antibody																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
D482 Tpl(7014)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	●	O	O	●	O	●
D504 Tpl(7014)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	O	O	O	O	O	●
D482 Tpl(7013)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	O	O	O	●	O	●
D504 Tpl(7013)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	●	O	O	●	O	●
D482 Tpl(6998)	●	O	O	●	●	●	●	●	●	●	●	●	●	O	O	O	O	●	O	●
D504 Tpl(6998)	●	O	O	●	●	●	●	●	●	●	●	●	●	O	O	O	O	●	O	●
D482 Tpl(6999)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	O	O	O	●	O	●
D504 Tpl(6999)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	O	O	O	●	O	●
D482 Tpl(7065)	●	O	O	●	●	●	●	●	●	●	●	●	●	O	O	O	O	O	O	●
D504 Tpl(7065)	●	O	O	●	●	●	●	●	●	●	●	●	●	O	O	O	O	O	O	●
D482 Tpp(Muguga)	●	●	●	●	●	●	●	●	●	●	●	●	●	●	O	O	O	O	O	O
D504 Tpp(Muguga)	●	●	●	●	●	●	●	●	●	●	●	●	●	●	O	O	O	O	O	O
D482 Tpp(Uganda)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	O	O	O	O	O	O
D504 Tpp(Uganda)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	O	O	O	●	O	O
D482 Tpp(Marikebuni)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	●	●	O	O	O	O
D504 Tpp(Marikebuni)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	●	●	O	O	O	O
D482 Tpp(Mariakani)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	●	O	O	●	O	O
D504 Tpp(Mariakani)	●	O	O	●	●	●	●	●	●	●	●	●	●	●	●	O	O	●	O	O
D482 Tpb(Boleni)	●	O	O	●	●	●	O	●	●	●	●	●	●	●	●	O	O	●	O	O
D504 Tpb(Boleni)	●	O	O	●	●	●	O	●	●	●	●	●	●	●	●	O	O	●	O	O

● Positive Reaction

0 Negative Reaction

(7014)-infected are different from the profiles of the other cell lines with MAb 18 and have identical profiles with one another with all the MAb. Only cells infected with the Tpl (6998) and Tpl (7065) were negative for MAb 14 but they differ from one another in that those infected with Tpl (6998) are positive for MAb 18. In certain cases a different profile was obtained when cells from different animals were infected with the same parasite stocks, for example D482 Tpl (7014) and D504 Tpl (7014) differed in their reactivity with MAb 15 and 18. D482 Tpl (7013) and D504 Tpl (7013) also differed in their reactivity with MAb 15. D482 cells infected with the parasite stock Tpl (7013) had a similar profile as cells infected with Tpl (6999), while D504 Tpl (7013) had a similar profile as D482 Tpl (7014).

Among the cell lines infected with the cattle-derived parasite stocks, the schizont antigen profiles were the same for PBM from different cattle infected with the same parasite stock except for those infected with Tpp (Uganda). The two cell lines, D504 Tpp (Uganda) and D482 Tpp (Uganda) differed in their reactivity with MAb 18.

3.1.2 IMMUNIZATION OF CATTLE

The 7 experimental cattle were immunized with buffalo-derived parasite stocks Tpl (6999), (6998), (7013), (7014) or (7065), or with the cattle-derived parasite stocks Tpb (Boleni) or Tpp (Uganda) by simultaneous inoculation of the stabilate and TCMN-LA and in some cases Clexon, as outlined in

Table 1. The cattle-derived stocks Tpb (Boleni) and Tpp (Uganda) were chosen to compare with the buffalo-derived stocks, since they have been previously reported to give protection against infection with a large variety of other parasite stocks (S. Morzaria, pers. comm.). PBM from cattle immunized with these stocks have not been previously tested in in vitro cytotoxicity assays. All 7 animals developed transient pyrexia and detectable parasitosis in the lymph node draining the site of inoculation. Parasitosis did not exceed 5% and was detectable for 1-12 days between 6-12 days after innoculation (Table 3).

3.2 CYTOTOXIC RESPONSE OF BULK CULTURES FROM IMMUNE ANIMALS

3.2.1 AUTOLOGOUS THEILERIA MIXED LEUCOCYTE REACTION

Peripheral blood mononuclear cells derived from blood collected in Alsevers solution or defibrinated with glass beads were assayed for proliferative responses to Theileria parva-infected cells in the MLR (Fig. 8) in order to determine the optimal conditions for stimulating PBM from the immune cattle. These conditions were then used to generate the cytotoxic cells which were tested for parasite strain specificity.

The PBM from all 7 immunized cattle were stimulated with irradiated T.parva-infected cells. The proliferative responses varied among animals but were depressed at high stimulator to responder ratios (Fig. 8). Strong proliferative responses were

TABLE 3 Reactions of cattle infected with buffalo-derived or cattle-derived T.parva parasite stocks

Animal no.	Parasite stock immunized with	Days to[a] schizont detection	Duration of schizont detection	Days to[b] temperature	Days to recovery	Severity[c] of reaction
D482	Tpl (7014)	11	1	-	-	mild
D487	Tpb (Boleni)	12	4	2	18	mild
D494	Tpl (6999)	7	8	10	18	mild
D503	Tpp (Uganda)	12	5	-	18	mild
D504	Tpl (6998)	7	10	11	19	moderate
D505	Tpl (7013)	11	3	3	18	mild
D508	Tpl (7065)	6	12	15	21	mild

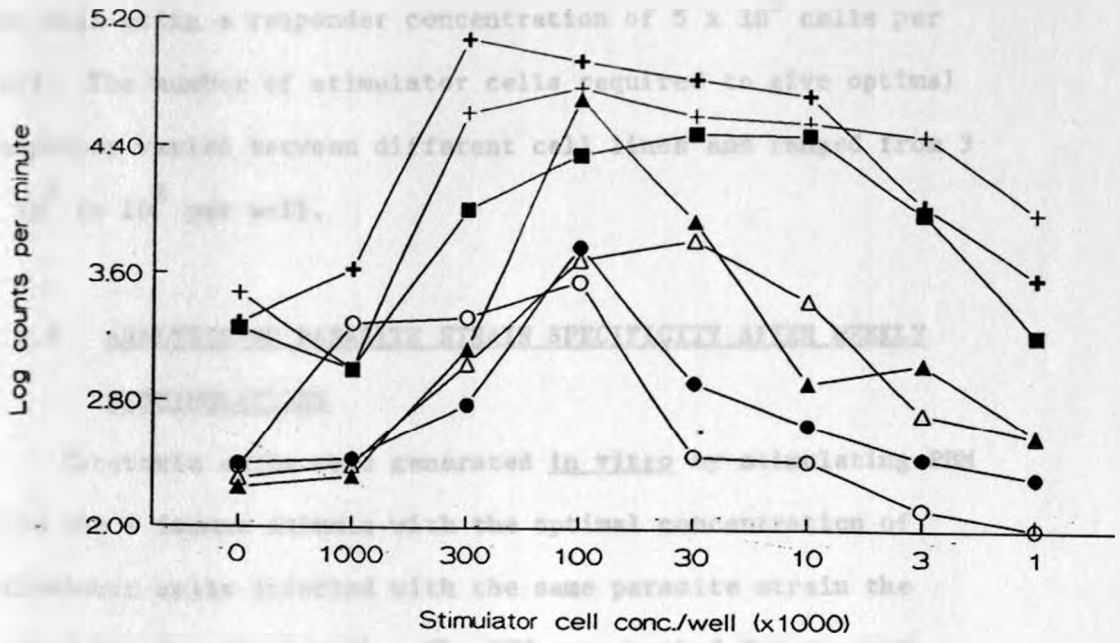
a All cattle were monitored daily by taking lymph node biopsies from day 5 onwards.

b All cattle were monitored daily by taking rectal temperatures from day 5 onwards.

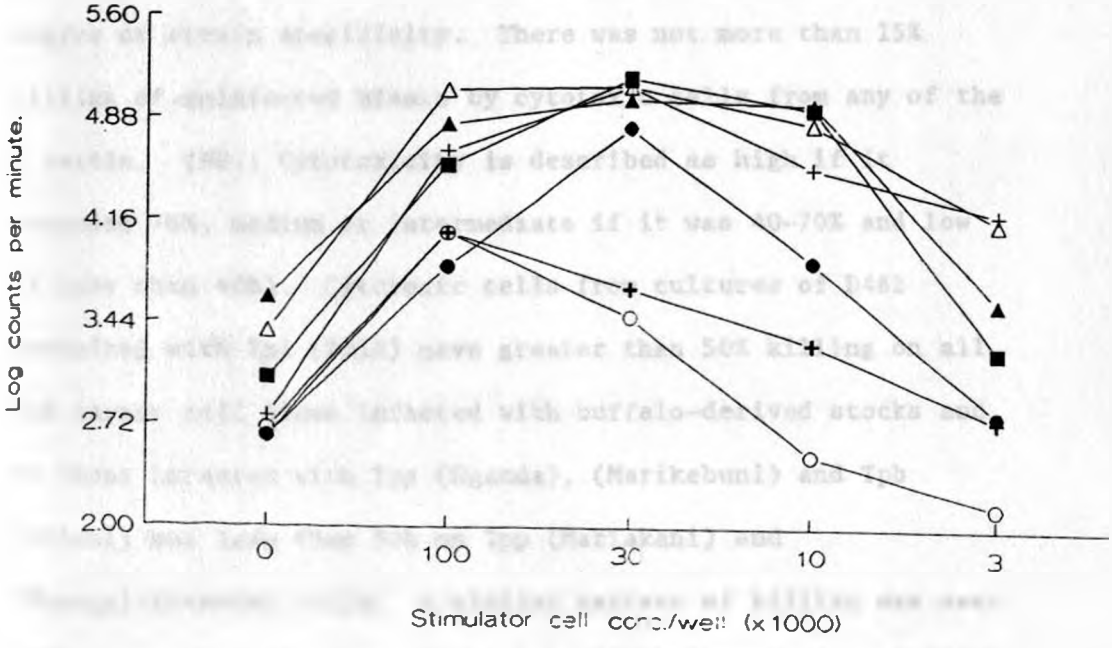
c A reaction was classified as mild when schizonts were detectable in lymph nodes for only a few days and when a febrile response was either absent or transient. A reaction was classified as moderate when a marked febrile response was observed and schizonts were present in substantial numbers in peripheral lymph nodes.

Fig. 8 Influence of stimulator cell concentration on the proliferative response of (a) Alsevers and (b) defibrinated PBM (5×10^5 cell/well) incubated for 5 and 6 days respectively. PBM were from animal no. D482 stimulated with Tp1 (7014) (closed triangles), D505 stimulated with Tp1 (7013) (closed squares), D504 stimulated with Tp1 (6998) (light crosses), D503 stimulated with Tpp (Uganda) (bold crosses), D494 stimulated with Tp1 (6999) (closed circles), D508 stimulated with Tp1 (7065) (open triangles) and D487 stimulated with Tpb (Boleni) (open circles). Proliferation of cells was measured by the incorporation of IUDR over 8 hr. Culture vessels were 96-well flat-bottom microtitre plate. All the stimulator cells were gamma-irradiated (5000 rads from a ^{137}Caesium source).

(a)


5.20
4.40
3.60
2.80
2.00
Log counts per minute
0
1000
300
100
30
10
3
1
Stimulator cell conc./well (x1000)

(b)


5.60
4.88
4.16
3.44
2.72
2.00
Log counts per minute.
0
100
30
10
3
Stimulator cell conc./well (x1000)

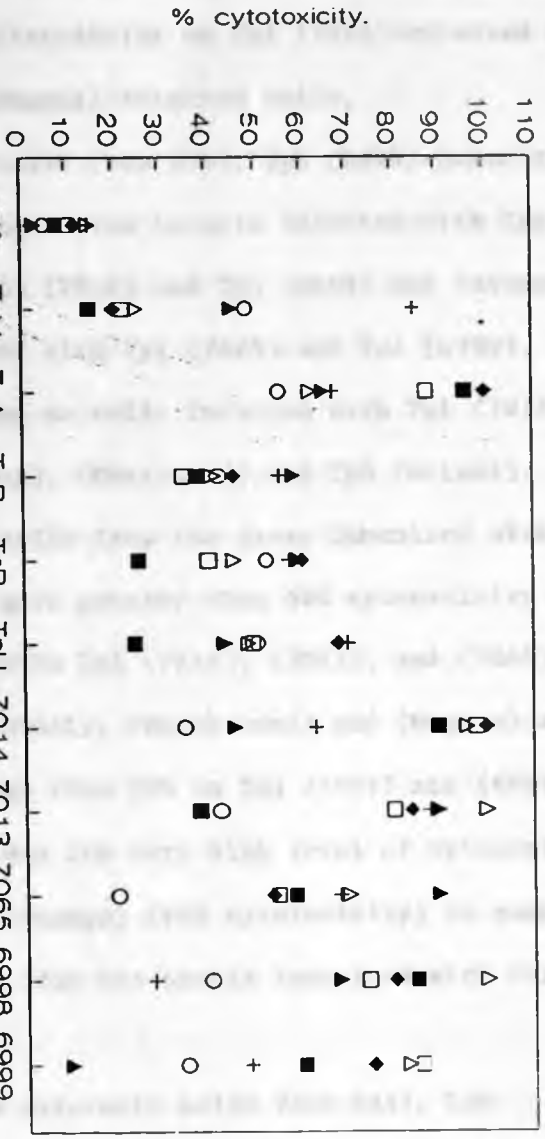
only obtained with 3 X 10^5 or fewer infected stimulator cells per well using a responder concentration of 5 x 10^5 cells per well. The number of stimulator cells required to give optimal responses varied between different cell lines and ranged from 3 x 10^5 to 10^4 per well.

3.2.2 ANALYSIS OF PARASITE STRAIN SPECIFICITY AFTER WEEKLY RESTIMULATIONS

Cytotoxic cells were generated *in vitro* by stimulating PBM from the 7 immune animals with the optimal concentration of stimulator cells infected with the same parasite strain the animal was immunized with. The PBM were tested for parasite specificity on a panel of target cells after 2, 3 or 4 stimulations *in vitro*.

The results show that the maximum cytotoxic response from all 7 animals (summarized in Fig. 9) did not exhibit a high degree of strain specificity. There was not more than 15% killing of uninfected blasts by cytotoxic cells from any of the 7 cattle. (NB.: Cytotoxicity is described as high if it exceeded 70%, medium or intermediate if it was 40-70% and low if less than 40%). Cytotoxic cells from cultures of D482 immunized with Tp1 (7014) gave greater than 50% killing on all the target cell lines infected with buffalo-derived stocks and on those infected with Tpp (Uganda), (Marikebuni) and Tpb (Boleni) but less than 50% on Tpp (Mariakani) and (Muguga)-infected cells. A similar pattern of killing was seen with cytotoxic cells from D505, Tp1 (7013)-immunized, and D508,

Fig. 9 Highest cytotoxic response of PBM from the 7 immune animals after 2, 3 or 4 stimulations *in vitro* at an E:T ratio of 40:1 or lower: D504 (closed triangles) stimulated with Tpl 6998, D503 (light crosses) stimulated with Tpp (Uganda), D487 (open circles) stimulated with Tpb (Boleni), D482 (closed diamond) stimulated with Tpl (7014), D494 (closed squares) stimulated with Tpl (6999), D505 (open squares) stimulated with Tpl (7013) and D508 (open triangle) stimulated with Tpl (7065). Cytotoxic responses were assayed in an ^{111}In-release assay on the panel of *T.parva*-infected target cells and uninfected blast cells. TpM is Tpp (Muguga), TpU is Tpp (Uganda), TpA is Tpp (Marikebuni), TpR is Tpp (mariakani) and TpB is Tpb (Boleni); 7014, 7013, 7065, 6998 and 6999 are *T.p.lawrencei* parasites from the appropriate buffalo.


% cytotoxicity.
0
10
20
30
40
50
60
70
80
90
100
110
unif-
TpM
TpA
TpR
TpB
TpU
7014
7013
7065
6998
6999
Parasite in target cells.

Tpl (7065)-immunized. Cytotoxic cells from cultures of PBM from D504, Tpl (6998)-immunized, were also similar except that they did not kill target cells infected with Tpl (6999) and had less than 50% cytotoxicity on Tpl (7014)-infected cells and was higher on Tpp (Muguga)-infected cells.

Cytotoxic cells from D494, Tpl (6999)-immunized, had high levels of killing on the targets infected with Tpp (Marikebuni), Tpl (7014) and Tpl (6998) and intermediate levels on those infected with Tpl (7065) and Tpl (6999). It had low levels of killing on cells infected with Tpl (7013), Tpp (Uganda), (Muguga), (Mariakani) and Tpb (Boleni).

Cytotoxic cells from the steer immunized with Tpp (Uganda), D503 gave greater than 50% cytotoxicity on the target cells infected with Tpl (7014), (7013), and (7065), and Tpp (Uganda), (Mariakani), (Marikebuni) and (Muguga) and Tpb (Boleni) but less than 50% on Tpl (6998) and (6999). The most marked feature was its very high level of cytotoxicity against the target Tpp (Muguga) (85% cytotoxicity) in comparison with cytotoxic cells from the cattle immunized with buffalo-derived parasites.

Killing by cytotoxic cells from D487, Tpb (Boleni)-immunized, was between 40 and 60% on all the infected target cell lines, except for those infected with Tpl (7065),(7014) and (6999) which were about 20 to 40%.

Restimulation of PBM from the immune animals resulted in an increase in cytotoxic activity of the cultures. Cytotoxic activity was maximal after 2 or 3 stimulations against the

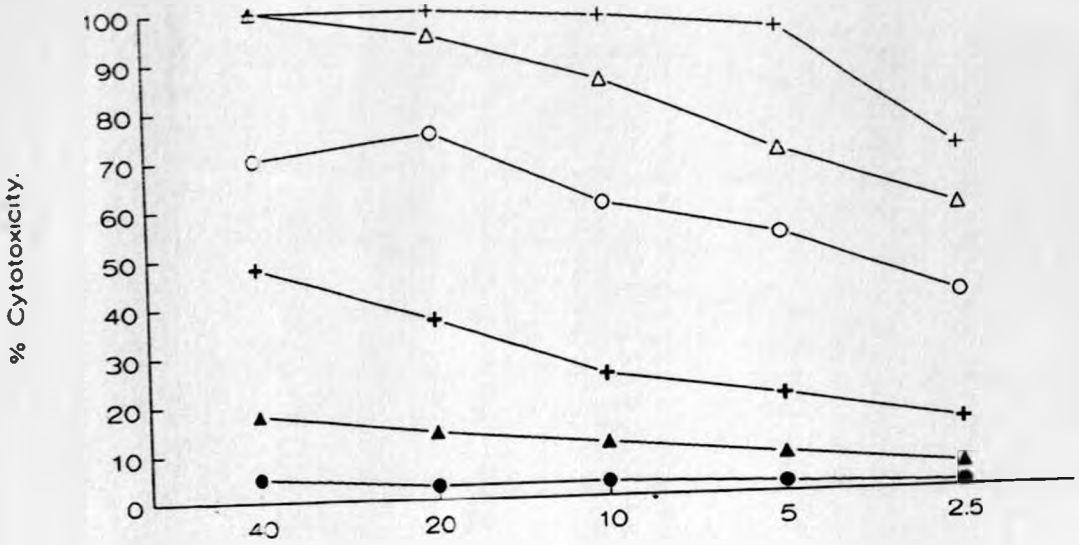
majority of targets cell lines including those which had been used as the stimulator cells to generate the bulk cultures. Cytotoxicity generally decreased against all targets after 4 stimulations (Figs. 10, 11 and 12). In some instances cytotoxic activity against cell lines infected with parasites other than the immunizing strain increased after 4 stimulations (Fig. 12c, target cell lines Tpl (7014) and Tpp (Marikebuni)). Cytotoxic activity against some target cell lines did not increase even after 4 stimulations (Figs. 10 and 11, target cell line Tpp (Muguga).

Although the parasite specitity did not change significantly for bulk cultures of PBM from 6 of the immunized cattle following weekly stimulations, (Fig.10-12, and results not shown for animals D487, D494 and D504) cytotoxic cells from cultures generated from PBM of the Tpl (7065)-immunized animal, D508, gave high killing on target Tpp (Muguga) after 4 stimulations (results not shown). This killing was however nonspecific since there was significant killing as well on uninfected blast cells. By contrast, cultures derived from the other immune animals did not give significant levels of cytotoxic killing on uninfected blasts (less than 10% at E:T ratio of 20/1) after 2, 3 or 4 restimulations.

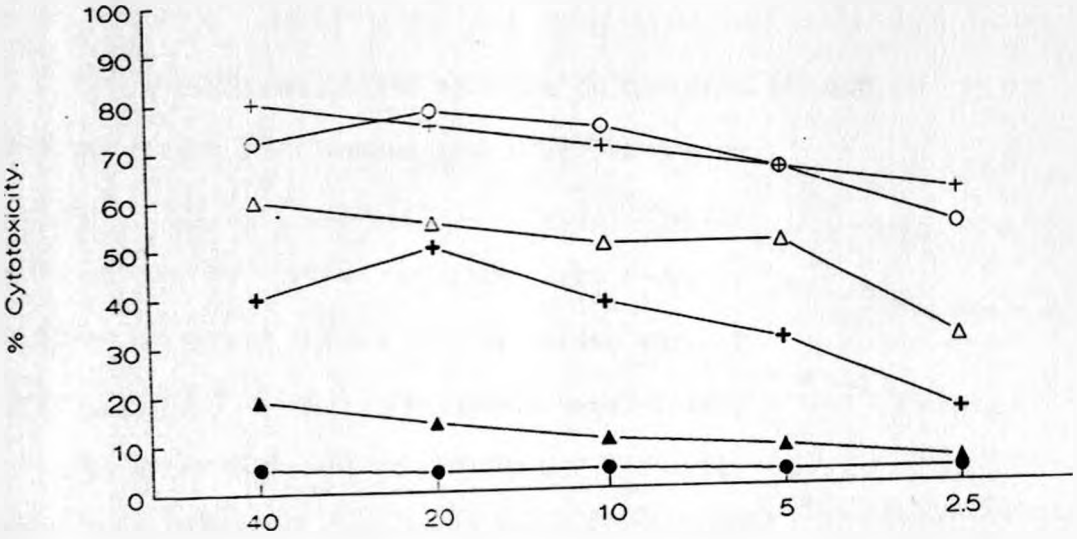
Fig. 10 Analysis of parasite strain specificity of cytotoxic cells from animal D505 stimulated with cells infected with Tp1 (7013) on autologous or BoLA-A-matched target cells infected with different buffalo or cattle-derived T.parva parasites: Tpp (Marikebuni) (light crosses), Tp1 (7014) (open triangles), Tp1 (7013) (open circles), Tpp (Uganda) (bold crosses), Tpp (Muguga) (closed triangles) and uninfected blasts (closed circles). They were tested after (a) 2 stimulations (b) 3 stimulations or (c) 4 stimulations in vitro by an ^{111}In-release cytotoxicity assay at an E:T ratio of 40:1 or lower.

D505

a


% Cytotoxicity.
100
90
80
70
60
50
40
30
20
10
0
40
20
10
5
2.5

b


% Cytotoxicity.
100
90
80
70
60
50
40
30
20
10
0
40
20
10
5
2.5

c

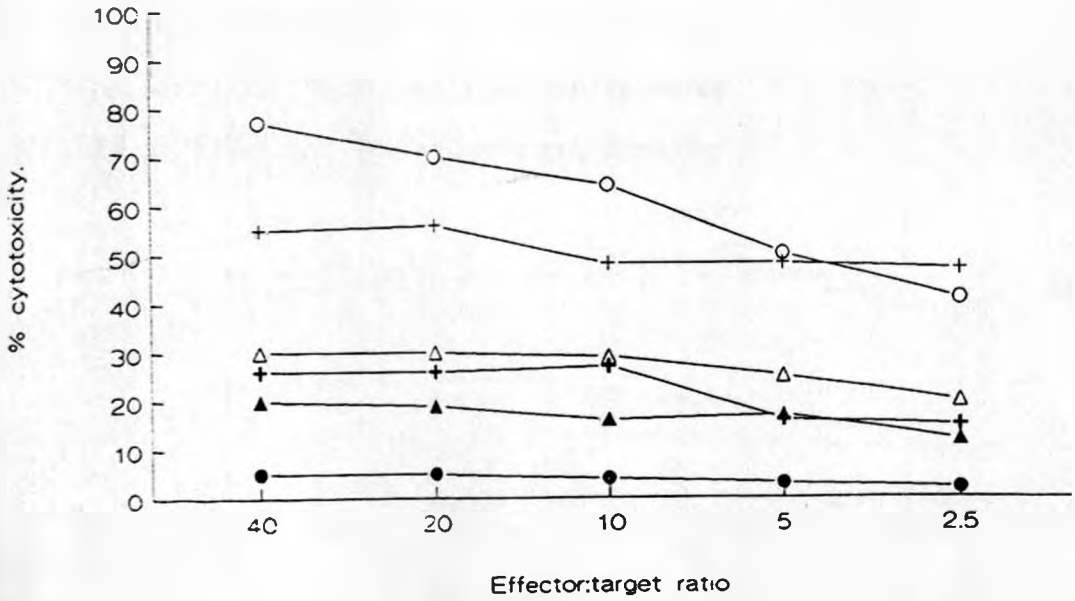
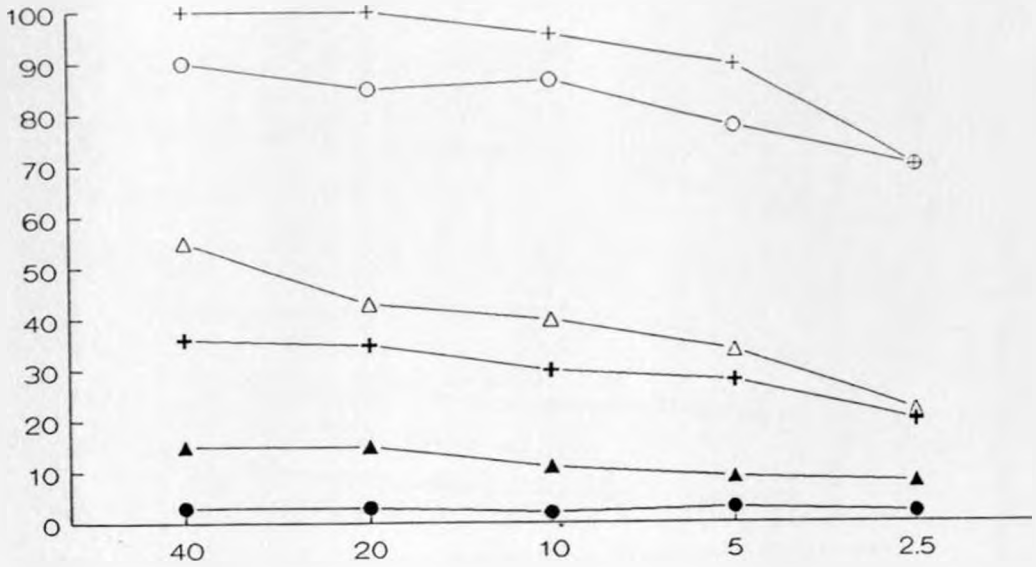

% cytotoxicity.
100
90
80
70
60
50
40
30
20
10
0
40
20
10
5
2.5
Effector:target ratio

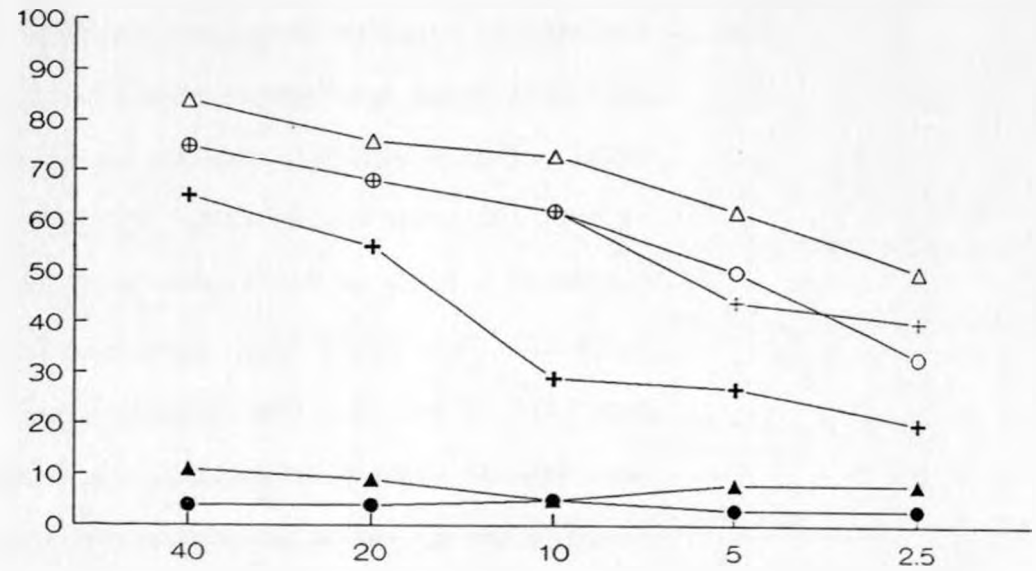
Fig. 11 Analysis of parasite strain specificity of cytotoxic cells from animal D482 stimulated with cells infected with Tpl (7014) on target cells infected with different buffalo or cattle-derived T.parva parasites: Tpl (7014) (light crosses), Tpl (7013) (open triangles), Tpp (Marikebuni) (open circles), Tpp (Uganda) (bold crosses), Tpp (Muguga) (closed triangles) and uninfected blasts (closed circles). They were tested after (a) 2 stimulations (b) 3 stimulations or (c) 4 stimulations in vitro in an ^{111}In-release cytotoxicity assay at an E:T ratio of 40:1 Or lower.

D482

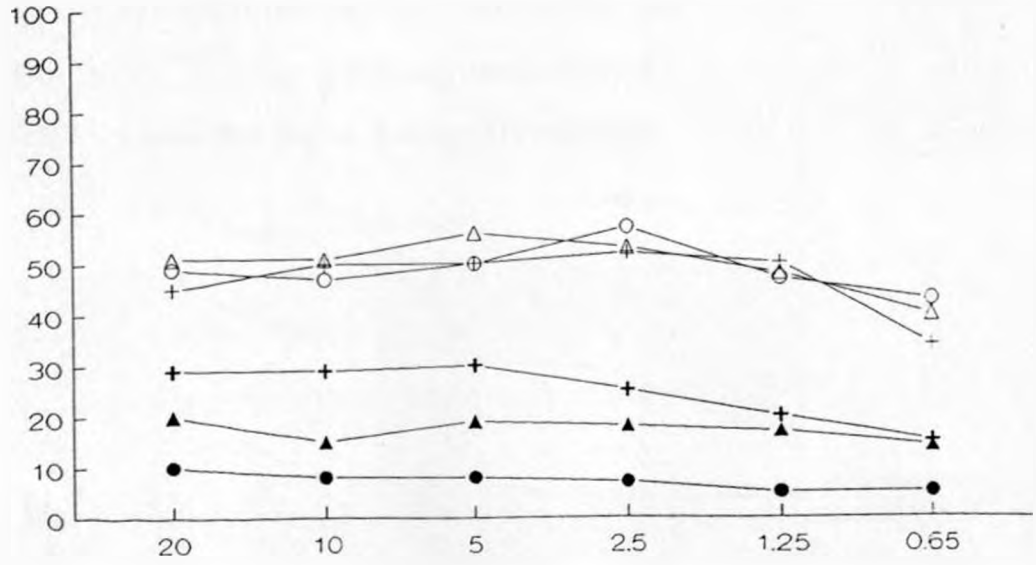
a



b



c

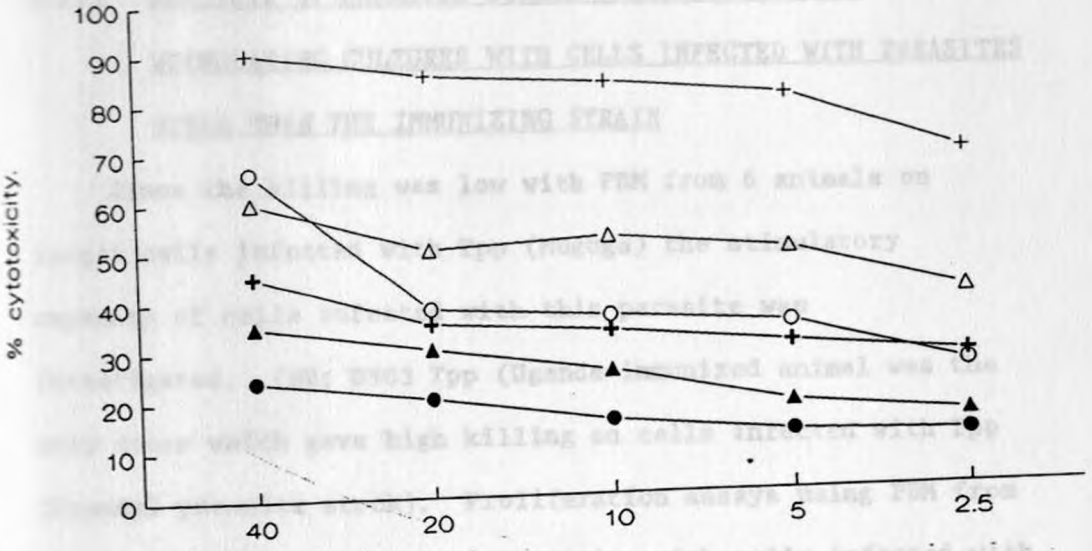


Effector:target ratio

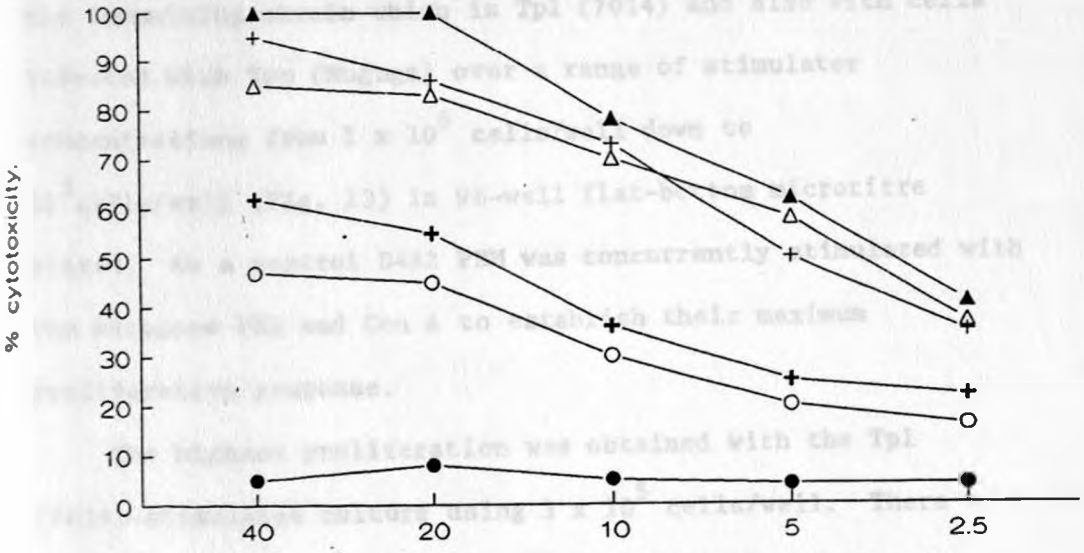
Fig. 12 Analysis of parasite strain specificity of cytotoxic cells from animal D503 stimulated with cells infected with Tpp (Uganda) on autologous or BoLA-A-matched target cells infected with different buffalo or cattle-derived T.parva parasites: Tpl (7014) (open circles), Tpl (7013) (open triangles), Tpp (Marikebuni) (bold crosses), Tpp (Uganda) (closed triangles), Tpp (Muguga) (light crosses) and uninfected blasts (closed circles). They were tested after (a) 2 stimulations (b) 3 stimulations or (c) 4 stimulations in vitro in an ^{111}In-release cytotoxicity assay at an E:T ratio of 40:1 or lower.

D503

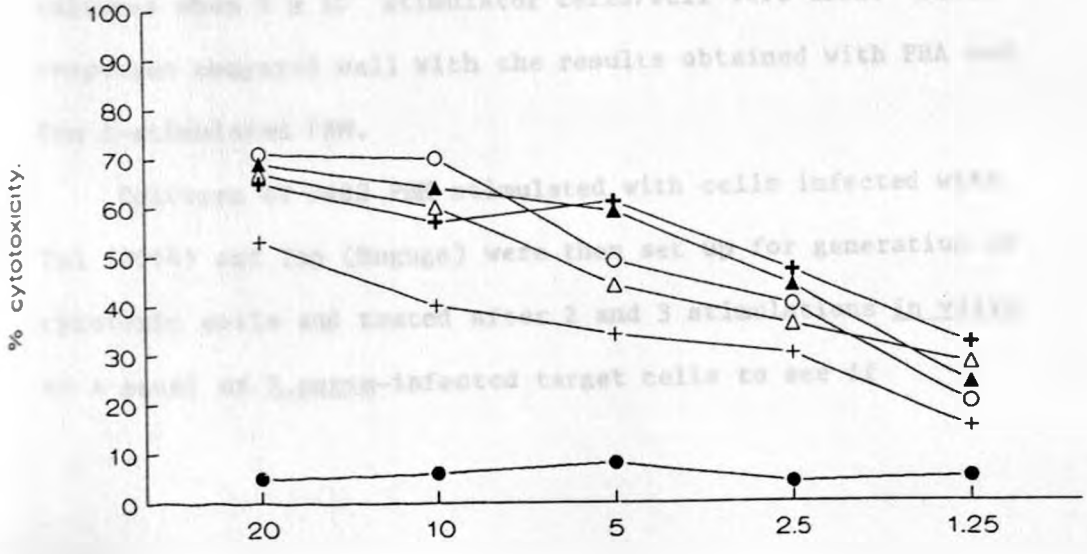
a


% cytotoxicity.
100
90
80
70
60
50
40
30
20
10
0
40
20
10
5
2.5

b


% cytotoxicity.
100
90
80
70
60
50
40
30
20
10
0
40
20
10
5
2.5

c


% cytotoxicity.
100
90
80
70
60
50
40
30
20
10
0
20
10
5
2.5
1.25
Effector:target ratio

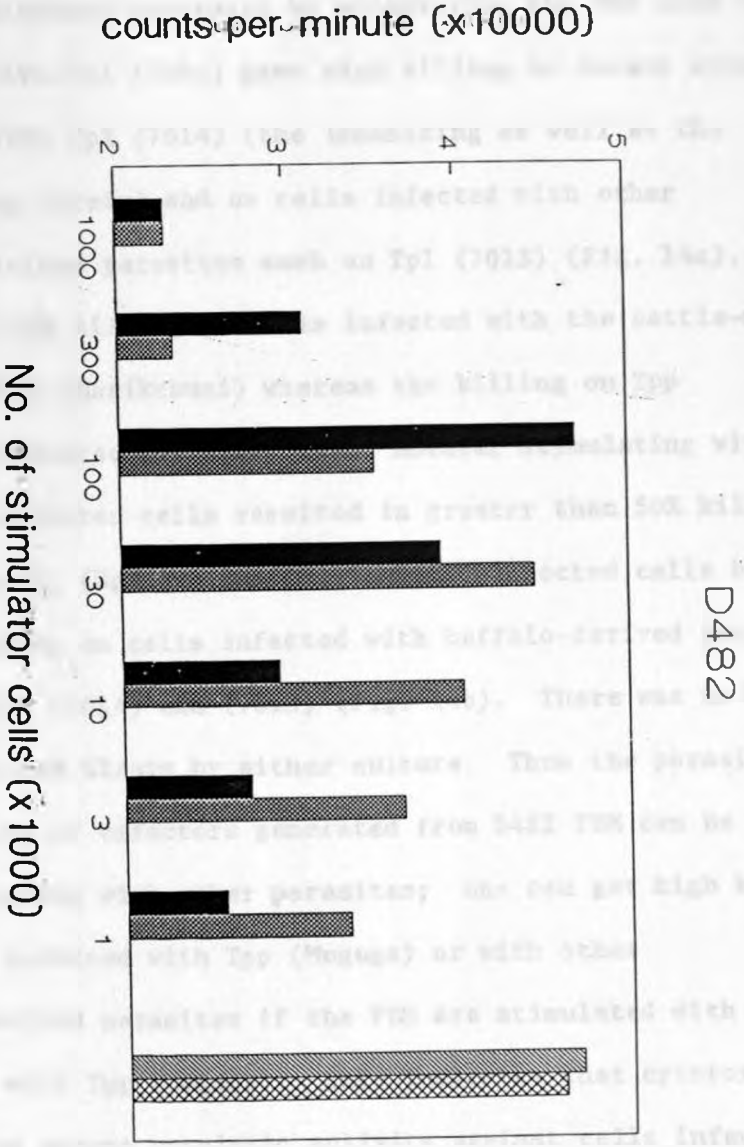
3.2.3 ANALYSIS OF PARASITE STRAIN SPECIFICITY AFTER STIMULATING CULTURES WITH CELLS INFECTED WITH PARASITES OTHER THAN THE IMMUNIZING STRAIN

Since the killing was low with PBM from 6 animals on target cells infected with Tpp (Muguga) the stimulatory capacity of cells infected with this parasite was investigated. (NB; D503 Tpp (Uganda-immunized animal was the only steer which gave high killing on cells infected with Tpp (Uganda) parasite stock). Proliferation assays using PBM from animal D482 were set up by stimulating with cells infected with the immunizing strain which is Tpl (7014) and also with cells infected with Tpp (Muguga) over a range of stimulator concentrations from 1×10^6 cells/well down to 10^3 cells/well (Fig. 13) in 96-well flat-bottom microtitre plates. As a control D482 PBM was concurrently stimulated with the mitogens PHA and Con A to establish their maximum proliferative response.

The highest proliferation was obtained with the Tpl (7014)-stimulated culture using 1×10^5 cells/well. There was comparable proliferation with Tpp (Muguga)-stimulated cultures when 5×10^4 stimulator cells/well were used. These responses compared well with the results obtained with PHA and Con A-stimulated PBM.

Cultures of D482 PBM stimulated with cells infected with Tpl (7014) and Tpp (Muguga) were then set up for generation of cytotoxic cells and tested after 2 and 3 stimulations *in vitro* on a panel of *T.parva*-infected target cells to see if

Fig. 13 Histogram of the proliferative response of PBM (5×10^5/well) to stimulation by different concentrations of cells infected with Tpl (7014) (closed bars) or with Tpp (Muguga) (small hatches) and of PBM (1.25×10^5/well) to the lectins Con A (diagonal lines) and PHA (bold hatches). Responder cells were Alsevers PBM and cultures were incubated in 96-well flat-bottom microtitre plates for 5 days. Proliferation was measured by incorporation of IUDR.

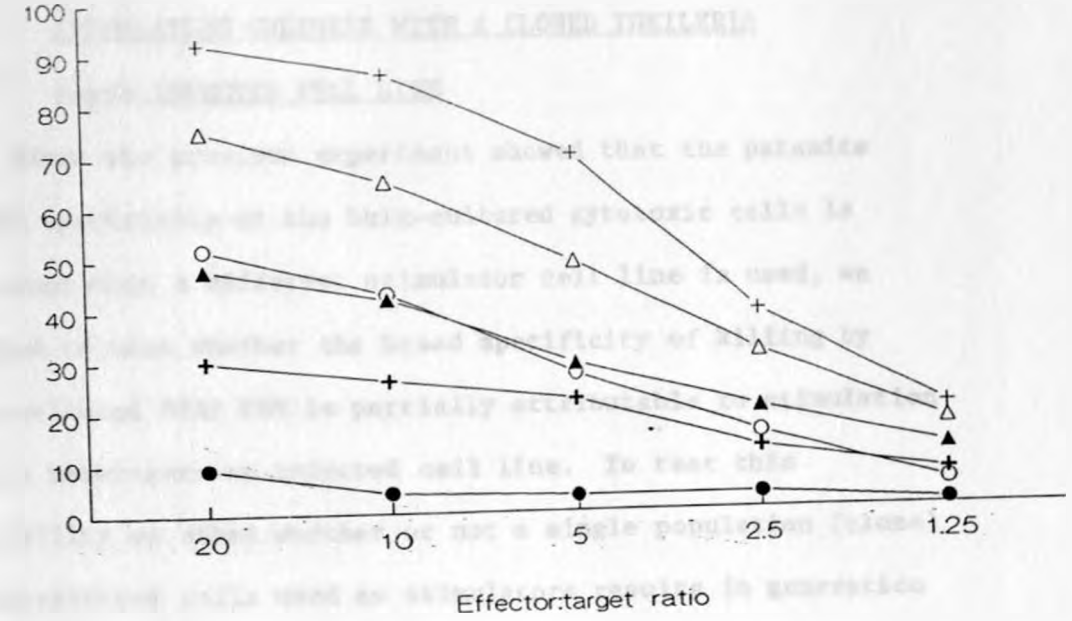

counts per minute (x10000)
2
3
4
5
1000
300
100
30
10
3
1
No. of stimulator cells (x1000)
D482

stimulation with cells infected with Tpp (Muguga) could increase the cytotoxic activity of PBM from cattle immunized with buffalo-derived parasites against Tpp (Muguga)-infected cells. Effectors generated by stimulating the PBM with cells infected with Tpl (7014) gave high killing on target cells infected with Tpl (7014) (the immunizing as well as the stimulating strain) and on cells infected with other buffalo-derived parasites such as Tpl (7013) (Fig. 14a). There was also high killing on cells infected with the cattle-derived parasite Tpp (Marikebuni) whereas the killing on Tpp (Muguga)-infected cells was low. However stimulating with Tpp (Muguga)-infected cells resulted in greater than 50% killing on Tpp (Muguga), (Uganda) and (Marikebuni)-infected cells but much lower killing on cells infected with buffalo-derived parasites such as Tpl (7014) and (7013) (Fig. 14b). There was no killing on uninfected blasts by either culture. Thus the parasite specificity of effectors generated from D482 PBM can be changed by stimulating with other parasites; one can get high killing on cells infected with Tpp (Muguga) or with other cattle-derived parasites if the PBM are stimulated with cells infected with Tpp (Muguga). This indicates that cytotoxic cells with potent cytolytic activity against cells infected with these cattle-derived parasites occur in the PBM of cattle immunized with buffalo-derived parasites.

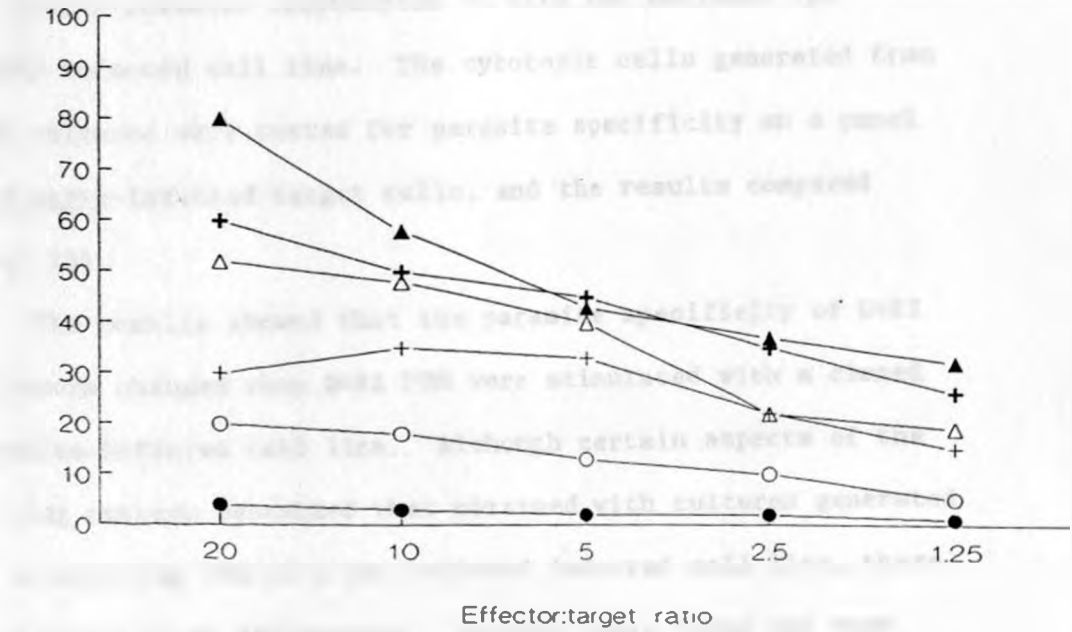
Fig. 14 Analysis of parasite strain specificity of PBM from animal D482 stimulated with cells infected with (a) Tpl (7014) or (b) Tpp (Muguga), and tested on autologous target cells infected with various buffalo or cattle-derived T.parva parasites: Tpl (7014) (light crosses), Tpp (Marikebuni) (open triangles), Tpl (7013) (open circles), Tpp (Muguga) (bold crosses), Tpp (Uganda) (closed triangles) and uninfected blasts (closed circles). They were tested after 3 stimulations in vitro in an ^{111}In-release cytotoxicity assay at an E:T ratio of 20:1 or lower.

D482

a


100
90
80
70
60
50
40
30
20
10
0
20
10
5
2.5
1.25
Effector:target ratio

b


100
90
80
70
60
50
40
30
20
10
0
20
10
5
2.5
1.25
Effector:target ratio

3.2.4 ANALYSIS OF PARASITE STRAIN SPECIFICITY AFTER STIMULATING CULTURES WITH A CLONED THEILERIA PARVA-INFECTED CELL LINE

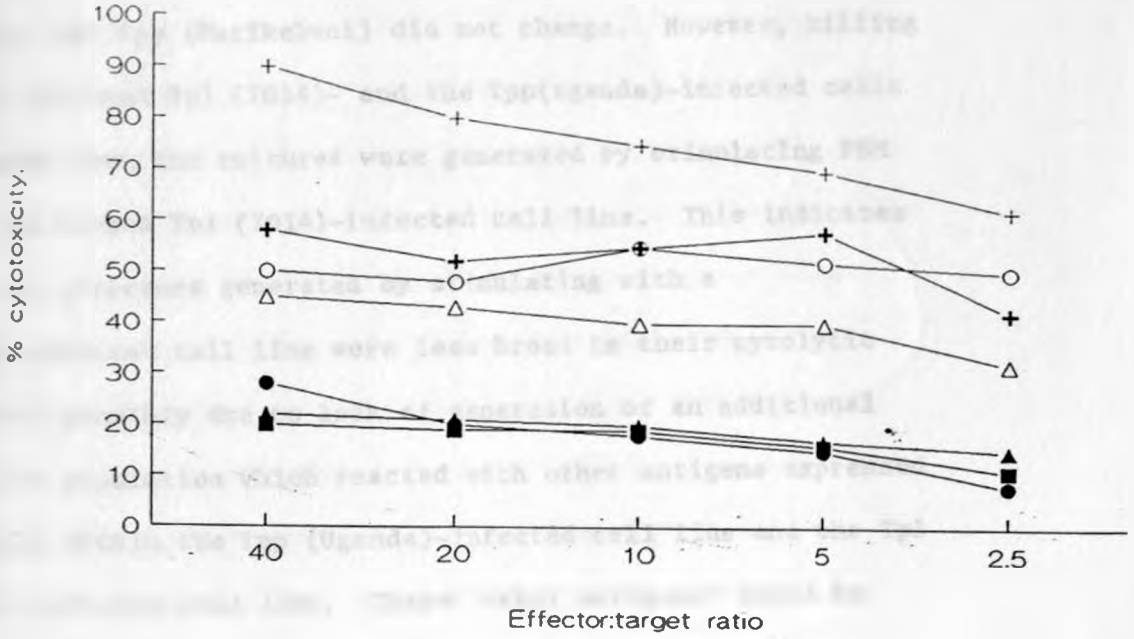
Since the previous experiment showed that the parasite strain specificity of the bulk-cultured cytotoxic cells is different when a different stimulator cell line is used, we decided to test whether the broad specificity of killing by bulk-cultured D482 PBM is partially attributable to stimulation with a heterogeneous infected cell line. To test this possibility we asked whether or not a single population (clone) of parasitized cells used as stimulators results in generation of an effector population that has a different parasite specificity from cultures stimulated with an uncloned infected cell line. To do this, PBM from animal D482, Tpl (7014)-immunized, were stimulated *in vitro* with a clone of D482 Tpl (7014)-infected lymphocytes or with the uncloned Tpl (7014)-infected cell line. The cytotoxic cells generated from both cultures were tested for parasite specificity on a panel of *T.parva*-infected target cells, and the results compared (Fig. 15).

The results showed that the parasite specificity of D482 effectors changed when D482 PBM were stimulated with a cloned parasite-infected cell line. Although certain aspects of the killing pattern resembled that obtained with cultures generated by stimulating PBM with the uncloned infected cell line, there were significant differences. In both cases there was very high killing on the cloned Tpl (7014)-infected cells and the

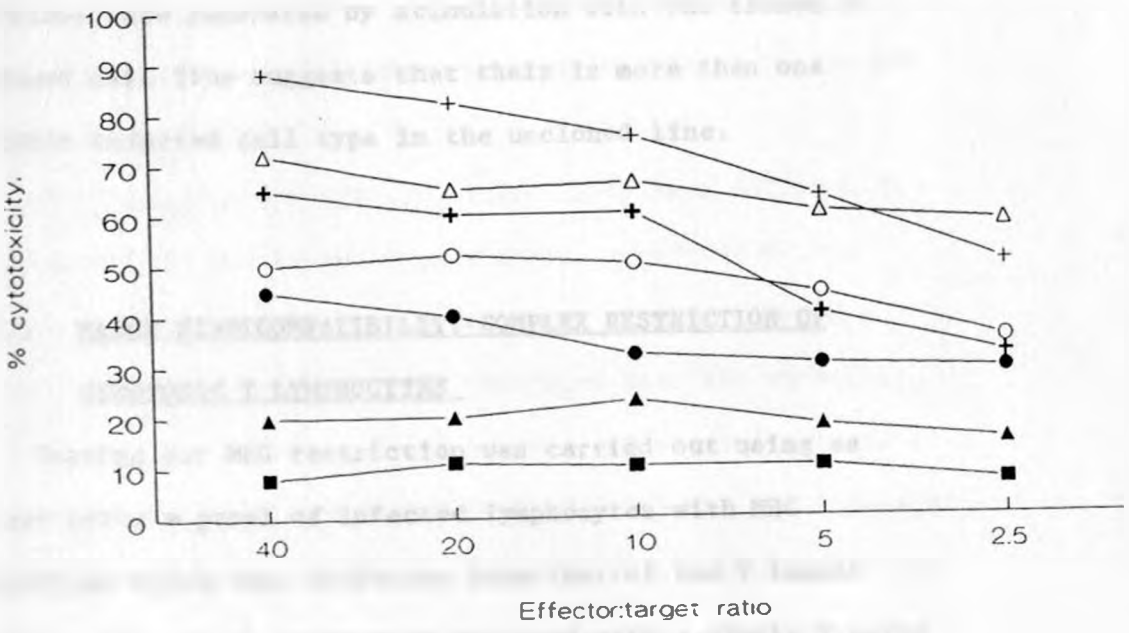
Fig. 15 Analysis of parasite strain specificity of PBM from animal D482 stimulated with (a) a clone of the Tp1 (7014)-infected cell line and (b) the uncloned Tp1 (7014)-infected cell line. They were tested on autologous or BOLA-A locus-matched target cells infected with different buffalo or cattle-derived T.parva parasites: clone of Tp1 (7014) (light crosses),Tp1 (7014) bulk (open triangles), Tp1 (7013) (open circles), Tpp (Muguga) (closed triangles), Tpp (Marikebuni) (bold crosses), Tpp (Uganda) (closed circles) and uninfected blasts (closed squares). They were tested after 3 stimulations in vitro in an ^{111}In-release cytotoxicity assay at an E:T ratio of 40:1 or lower.

D482

a


% cytotoxicity.
100
90
80
70
60
50
40
30
20
10
0
40
20
10
5
2.5
Effector:target ratio

b


% cytotoxicity.
100
90
80
70
60
50
40
30
20
10
0
40
20
10
5
2.5
Effector:target ratio

level of cytotoxicity against target cell-lines Tpl (7013), Tpp (Muguga) and Tpp (Marikebuni) did not change. However, killing on the uncloned Tpl (7014)- and the Tpp(uganda)-infected cells decreased when the cultures were generated by stimulating PBM with the cloned Tpl (7014)-infected cell line. This indicates that the effectors generated by stimulating with a cloned-infected cell line were less broad in their cytolytic activity possibly due to lack of generation of an additional effector population which reacted with other antigens expressed on cells within the Tpp (Uganda)-infected cell line and the Tpl (7014)-infected cell line. These "other antigens" could be representative of a distinct population within the uncloned target cell line, or of antigens expressed on all cells within the uncloned cell line but perhaps be more effective in engaging cytotoxic T cells. The fact that the level of killing on the cloned target cell line was equivalent whether the effectors were generated by stimulation with the cloned or uncloned cell line suggests that their is more than one distinct infected cell type in the uncloned line.

3.2.5 MAJOR HISOTCOMPATIBILITY COMPLEX RESTRICTION OF CYTOTOXIC T LYMPHOCYTES

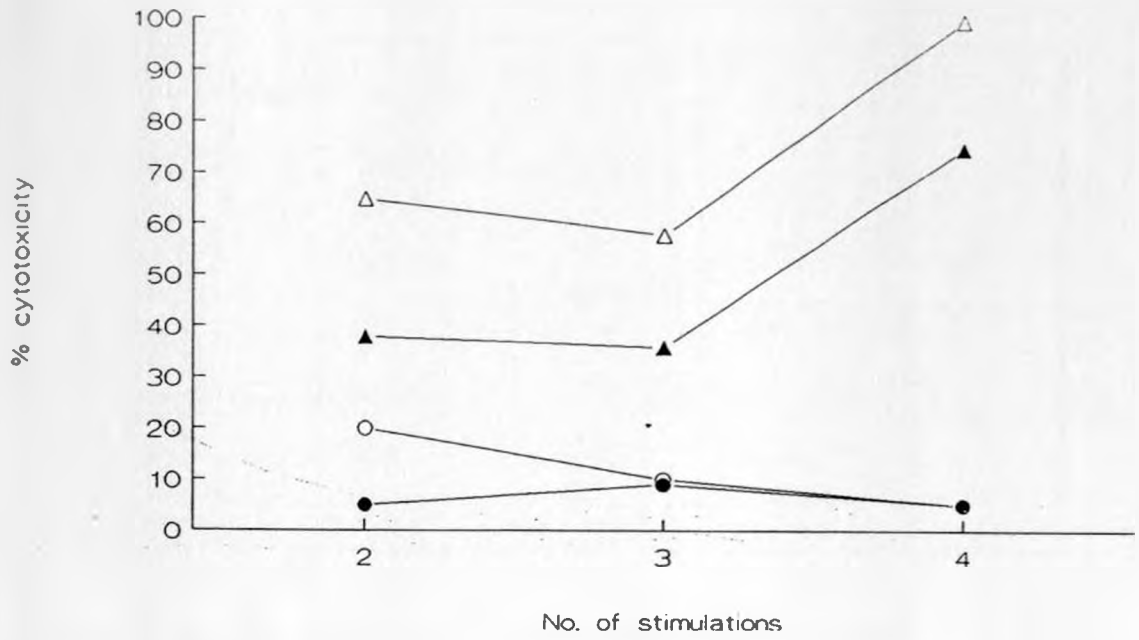
Testing for MHC restriction was carried out using as target cells a panel of infected lymphocytes with MHC haplotypes which were different from that of the 7 immune cattle. All target cells were infected with a single T.parva

parasite stock, Tpp (Marikebuni). The cell lines shared one, two or no BoLA A-locus class I specificities with the cytotoxic (effector) population being tested (section 2.2.7). The Theileria specific cytotoxic lymphocytes from animal D505, Tp1 (7013)-immunized, and D482, Tp1 (7014)-immunized, (both animals have the phenotype w6/KN8) (Fig. 16) gave the highest killing on the autologous (in the case of D482) or fully MHC-matched (in the case of D505) target cell line and the second highest killing was on the infected cell line w10/KN8 which shared the KN8 haplotype. There was little or no significant killing after the second restimulation on the half-matched w6/w7 cell line nor ever on the mismatched cell line (haplotype w10, KN104/W10, KN104). Thus, with increasing numbers of restimulations the proportion of cytotoxic cells restricted by KN8 became more dominant in the cultures. There was no killing on the MHC-matched uninfected blasts (data not shown). The parasite specific cytotoxic response of the bulk-cultured cells from these cattle was therefore largely restricted by the KN8 specificity.

The Theileria specific cytotoxic cells from cattle D487 and D504 which were of MHC type w6/KN12, were tested in a similar manner as their w6/KN8 siblings. The results on a panel of target cells (Fig. 17) indicated that the cytotoxic cells from D487 were restricted by KN12 and the majority of those from D504 were restricted by KN12, although the levels of cytotoxicity on both of the half-matched target cell lines was considerably lower than that on the autologous cell lines.

Fig. 16 Analysis of MHC restriction of Theileria-specific cytotoxic cells from animal D505 immunized with Tp1 (7013) and D482 immunized with Tp1 (7014) (both have the phenotype w6/KN8). Cytotoxic cells were tested on target cells with varying MHC haplotypes but that were infected with a single parasite stock (Tpp (Marikebuni)). They were fully matched for both BoLA A-locus phenotypes (open triangles), matched for the KN8 phenotype only (closed triangles), matched for the w6 phenotype only (open circles) or had neither of the A-locus phenotypes w6 or KN8 (closed circles). The PBM was tested after 2, 3 or 4 stimulations in vitro in an ^{111}In-release cytotoxicity assay.

D505


% cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
2
3
4
No. of stimulations

D482

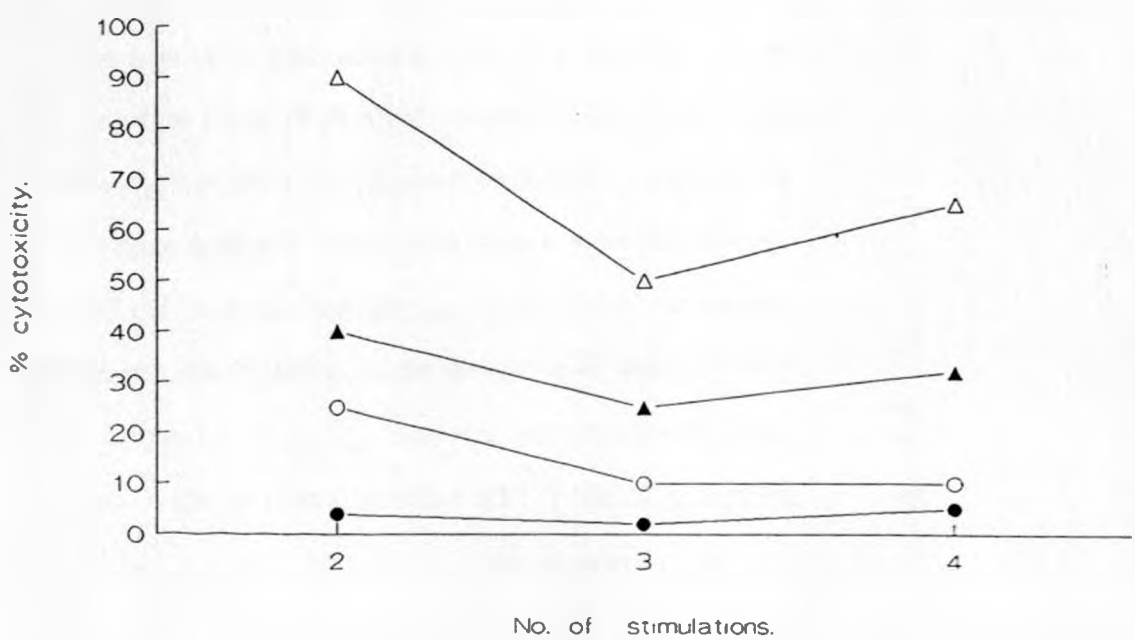
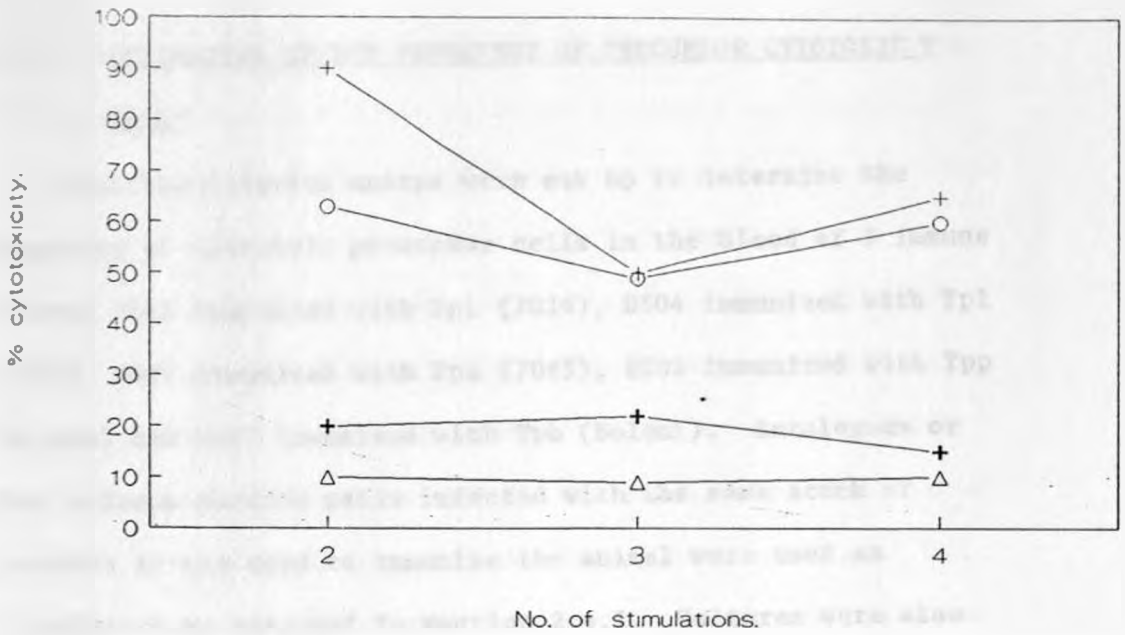
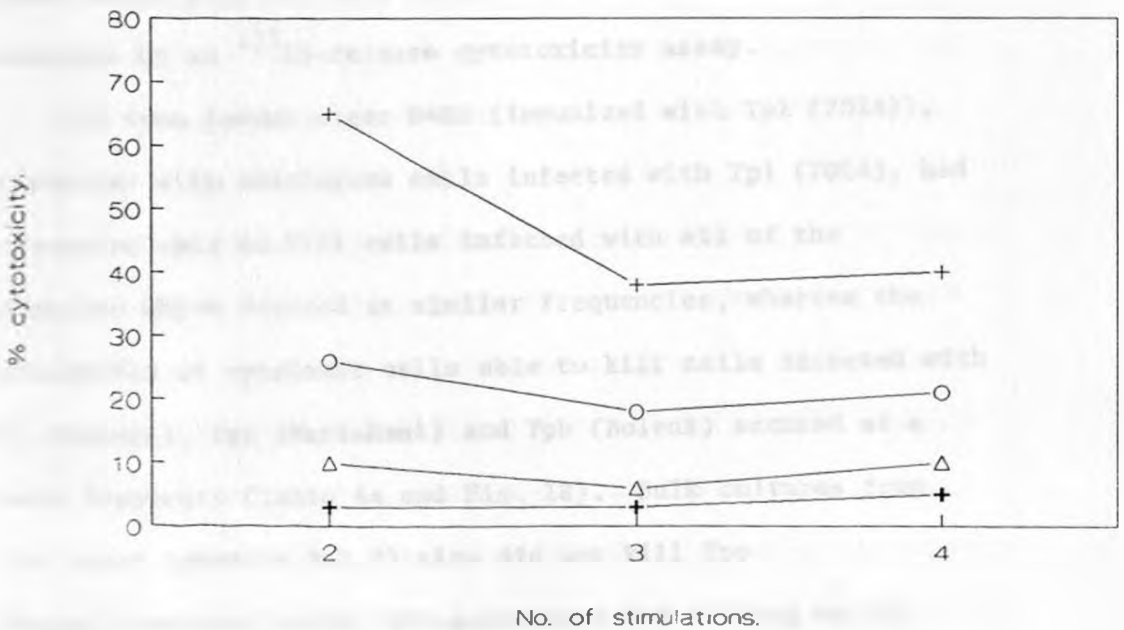

% cytotoxicity.
100
90
80
70
60
50
40
30
20
10
0
2
3
4
No. of stimulations.

Fig. 17 Analysis of MHC restriction of Theileria-specific cytotoxic cells from animal D487 immunized with Tpb (Boleni) and D504 immunized with Tpl (6998) (both animals have the phenotype w6/KN12). Cytotoxic cells were tested on target cells with varying MHC haplotypes but that were infected with a single parasite stock, Tpp (Marikebuni). They were fully-matched for both BoLA A locus phenotypes (light crosses), matched for the KN12 phenotype only (open circles), matched for the w6 phenotype only (open triangles) or had neither of the A-locus phenotypes w6 or KN12 (bold crosses). The PBM was tested after 2, 3 or 4 stimulations in vitro by an ^{111}In release cytotoxicity assay.

D487


100
90
80
70
60
50
40
30
20
10
0
% cytotoxicity.
2
3
4
No. of stimulations.

D504


80
70
60
50
40
30
20
10
0
% cytotoxicity.
2
3
4
No. of stimulations.

3.3 LIMITING DILUTION ANALYSIS

3.3.1 ESTIMATION OF THE FREQUENCY OF PRECURSOR CYTOTOXIC T CELLS

Limiting dilution assays were set up to determine the frequency of cytotoxic precursor cells in the blood of 5 immune steers, D482 immunized with Tp1 (7014), D504 immunized with Tp1 (6998), D508 immunized with Tp1 (7065), D503 immunized with Tpp (Uganda) and D487 immunized with Tpb (Boleni). Autologous or BoLA A-locus-matched cells infected with the same stock of parasite as was used to immunize the animal were used as stimulators as outlined in section 2.4.1. Cultures were also set up with PBM from some of these steers by stimulating with cells infected with T.parva parasites other than immunizing stock. Cultures were assayed for cytotoxicity on a panel of target cells infected with cattle- or buffalo-derived T.parva parasites by an ^{111}In-release cytotoxicity assay.

PBM from immune steer D482 (immunized with Tp1 (7014)), stimulated with autologous cells infected with Tp1 (7014), had precursors able to kill cells infected with all of the parasites which occured at similar frequencies, whereas the frequencies of cytotoxic cells able to kill cells infected with Tpp (Muguga), Tpp (Mariakani) and Tpb (Boleni) occured at a lower frequency (Table 4a and Fig. 18). Bulk cultures from this steer (section 3.2.2) also did not kill Tpp (Muguga)-infected cells, but gave about 50% killing on Tpp (Mariakani)-infected cells and were highly cytotoxic (greater

than 50% killing) for all the other target cell lines including Tpb (Boleni)-infected cells. The Tpl (7013)-infected cell line was more sensitive as a target than Tpl (7014) since cultures stimulated with cells infected with Tpl (7014) and tested on both Tpl (7014) and (7013)-infected target cells had some wells containing effector cells which could kill the Tpl (7013)-infected target cells but not the Tpl (7014)-infected target cells. When PBM from D482 were stimulated with cells infected with Tpl (7013) the frequency of effectors cytotoxic for cells infected with Tpl (7014) was equal to or higher than when the PBM had been stimulated with cells infected with Tpl (7014), whereas the frequency was increased for cells infected with Tpl (7013). However, when PBM from steer D482 were stimulated with cells infected with Tpp (Muguga) and tested on that cell line, the frequency of cytotoxic effectors specific for Tpp (Muguga)-infected cells was greatly increased, suggesting that the effector population recognizing an antigen common between Tpp (Muguga) and Tpl (7014)-infected cells was preferentially stimulated.

PBM from immune steer D504 immunized with Tpl (6998) had a high frequency of effectors specific for Tpp (Marikebuni) and Tpl (6999), a slightly lower frequency for cells infected with Tpp (Uganda), Tpb (Boleni), Tpl (7014) and Tpl (7013), and the lowest frequency for cells infected with the immunizing strain Tpl (6998) and for Tpl (7065), Tpp (Muguga) and Tpp (Mariakani) (Table 4b and Fig. 18). The result for cells infected with the immunizing strain Tpl (6998) tended to be inconsistent but the

graphed result (1/f = 7142) was the more statistically correct (R^2 value = 0.856). In addition, PBM from this animal stimulated with Tpl (7014)-infected cells and tested on targets infected with Tpl (6998)-infected cells gave a similar result (1/f = 7692), although the frequency of effectors was increased for Tpl (7014)-infected cells as expected (1/f = 1330). Bulk cultures from this animal gave high cytotoxic activity on the target cell line infected with Tpl (7065) and gave no killing on Tpl (6999)-infected cells. This latter result is however inconclusive since the cultures were only tested once on this cell line, but both of the bulk culture results contrast sharply with the results obtained in the LDA with the same target cell lines.

PBM from immune steer D508 immunized with Tpl (7065) had a high frequency of effectors specific for cells infected with Tpl (7065) and Tpp (Marikebuni), a moderate frequency for those infected with Tpp (Mariakani), Tpp (Uganda), Tpb (Boleni), and Tpl (6998), (6999) and (7013), but a low frequency for cells infected with Tpp (Muguga) and Tpl (7014) (Table 4c and Fig. 18). Bulk cultures from this animal had cytolytic activity on all target cell lines except Tpp (Muguga)-infected cells, and in fact gave high killing on Tpl (7014)-infected cell line, and in this respect differed from the LDA result. The killing by the restimulated bulk-cultured cells was moderate on Tpp (Mariakani), Tpp (Uganda) and Tpb (Boleni)-infected cells consistent with the results of the LDA. Cultures from D508 PBM stimulated with Tpl (6998)-infected cells and tested on Tpl

(7065)-infected cells resulted in a higher frequency of CTL able to kill Tpl (7065)-infected cell line (1/f = 1560) than for one of the results obtained when the stimulator cell line Tpl (6998) was used (1/f = 2530). Also stimulating with cells infected with Tpl (6998) and testing on cells infected with Tpl (7065) gave a higher frequency (1/f = 1560) than that obtained when this culture was tested on cells infected with Tpl (6998) (1/f = 2164).

PBM from immune steer D503 immunized with Tpp (Uganda) had a moderate frequency of effectors specific for cells infected with many of the T.parva parasite stocks including Tpp (Muguga), and the lowest frequency for cells infected with Tpl (6998) (Table 4d and Fig. 18). Bulk cultures from this steer also had high cytolytic activity on all the target cell lines except on Tpl (6998)-infected cells. Stimulating PBM with cells infected with Tpl (6998) in the LDA and testing these on Tpp (Uganda)-infected cells gave similar frequencies as those obtained when cultures were stimulated with Tpp (Uganda)-infected cells.

PBM from immune steer D487 immunized with Tpb (Boleni) had the highest frequency of effectors specific for Tpb (Boleni) and Tpp (Mariakani)-infected cells, a considerably lower frequency for Tpp (Muguga), (Uganda), (Marikebuni), and Tpl (7014)-infected cells and a very low frequency for cells infected with Tpl (7013), (6999), (6998), and (7065)-infected cells (Table 4e and Fig. 18). Bulk cultures from this animal gave low cytotoxic activity on Tpl (7065)-infected cells and moderate (about 50%) on all the rest.

TABLE 4a Estimation of the frequency of cytotoxic T-cells in the PBM of animal no. 0482 immunized with parasite stock Tpl (7014)[a]

Target cell	Stimulator Cell line					
	Tpl (7014)				Tpl (7013)	Tpp (Muguga)
	Test dates				Test date	Test date
	21.3	23.4	9.5	6.5	9.5	16.5
Tpp (Muguga)	13060[b]			9800(2305)		5464
Tpp (Marikebuni)		5500	6220(509)			
Tpp (Mariakani)		9850	14020(2948)			
Tpp (Uganda)		6420				
Tpb (Boleni)	12690					
Tpl (7014)	6000	5291	6944(829)		5291	
Tpl (7013)	6200		6289(63)		2645	
Tpl (6999)				6500		
Tpl (6998)				5930		
Tpl (7065)				7200		

a The PBM from this animal were stimulated once in vitro, with cells infected with the same T. parva parasite stock as was used for immunization, or with another cell line believed to be antigenically different from the immunizing strain, and tested against the panel of target cell lines.

b The reciprocal of the precursor frequency of cytotoxic T-cells. The value in parenthesis is the standard deviation where more than one result was statistically valid.

TABLE 4b Estimation of the frequency of cytotoxic T-cells in the PBM of animal no. D504 immunized with parasite stock Tpl (6998)[a]

Target cell	Stimulator Cell line: Tpp (6998) Test dates 10.5	17.8	25.8	26.8	Tpl(7014) Test Date 10.8
Tpp (Muguga)				8500[b]	
Tpp (Marikebuni)		2500			
Tpp (Mariakani)				9345	
Tpp (Uganda)		4800			
Tpb (Boleni)			4318(72)	4420	
Tpl (7014)	1472	4440(2098)			1330
Tpl (7013)			4230		
Tpl (6999)				2540	
Tpl (6998)		16666	7142		7692
Tpl (7065)			7100		

[a] The PBM from this animal were stimulated once *in vitro*, with cells infected with the same *T. parva* parasite stock as was used for immunization, or with another cell line believed to be antigenically different from the immunizing strain, and tested against the panel of target cell lines.

[b] The reciprocal of the precursor frequency of cytotoxic T-cells. The value in parenthesis is the standard deviation where more than one result was statistically valid.

TABLE 4c Estimation of the frequency of cytotoxic T-cells in the PBM of animal no. D508 immunized with parasite stock Tpl (7065)[a]

Target cell	Stimulator Cell line: Tpl (7065) Test dates 18.5	19.5	21.5	24.5	Tpl (6998) Test date 18.5
Tpp (Muguga)			12500[b]		
Tpp (Marikebuni)		2100(381)		1560	
Tpp (Mariakani)		5494			
Tpp (Uganda)				6200	
Tpb (Boleni)				5800	
Tpl (7014)		11000(353)	10500		
Tpl (7013)		5000			
Tpl (6999)			4120		
Tpl (6998)	4224				2164
Tpl (7065)	1250(905)			2530	1560

a The PBM from this animal were stimulated once in vitro, with cells infected with the same T. parva parasite stock as was used for immunization, or with another cell line believed to be antigenically different from the immunizing strain, and tested against the panel of target cell lines.

b The reciprocal of the precursor frequency of cytotoxic T-cells. The value in parenthesis is the standard deviation where more than one result was statistically valid.

TABLE 4d Estimation of the frequency of cytotoxic T-cells in the PBM of animal no. D503 immunized with parasite stock Tpp (Uganda)[a]

Target cell	Stimulator Cell line: Tpp (Uganda) Test dates 11.5	13.9	15.9	Tpl (6998) Test date 11.5
Tpp (Muguga)		6030[b]		
Tpp (Marikebuni)		5500		
Tpp (Mariakani)			6845	
Tpp (Uganda)	3460(608)			4320
Tpb (Boleni)			6230	
Tpl (7014)		6624		
Tpl (7013)			4315	
Tpl (6999)		5920		
Tpl (6998)	7940			5850
Tpl (7065)			4012	

a The PBM from this animal were stimulated once in vitro, with cells infected with the same T. parva parasite stock as was used for immunization, or with another cell line believed to be antigenically different from the immunizing strain, and tested against the panel of target cell lines.

b The reciprocal of the precursor frequency of cytotoxic T-cells. The value in parenthesis is the standard deviation where more than one result was statistically valid.

TABLE 4e Estimation of the frequency of cytotoxic T-cells in the PBM of animal no. D487 immunized with parasite stock Tpb (Boleni)[a]

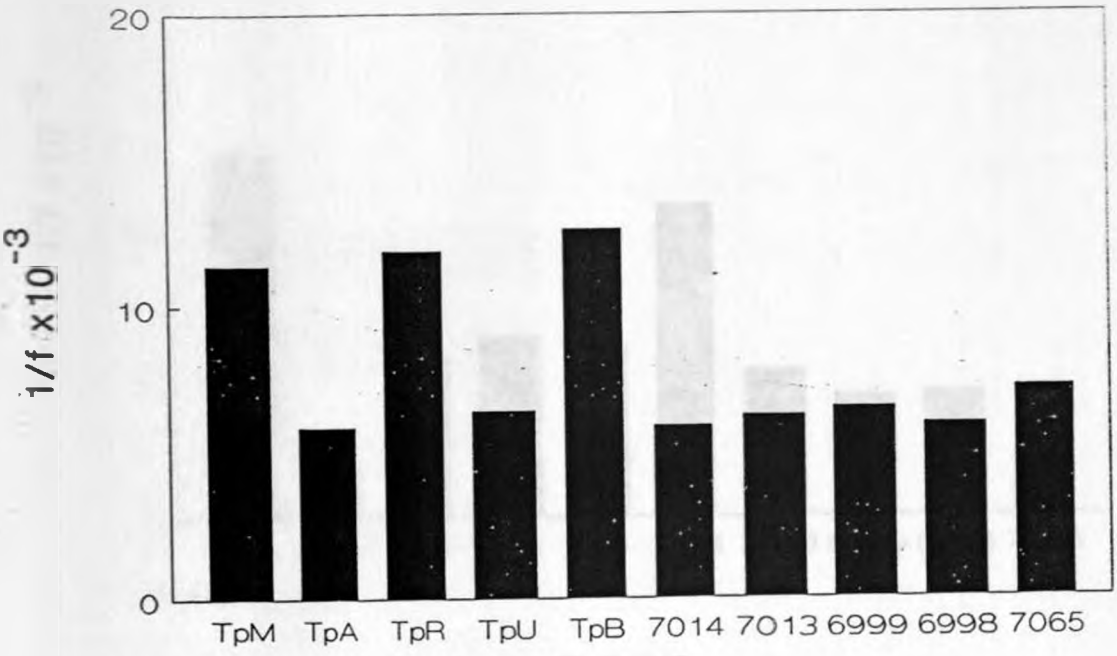
Target cell	Stimulator Cell line					
	Tpb (Boleni)				Tpl (7014)	Tpl (6998)
	Test dates				Test date	Test date
	20.5	25.8	7.9	9.9	20.5	25.8
Tpp (Muguga)				9976[b]		
Tpp (Marikebuni)			9540			
Tpp (Mariakani)			1438			
Tpp (Uganda)				7850		
Tpb (Boleni)	2590	(646)	3012	1742	8970	4350
Tpl (7014)	5347	8493(2224)			6493	
Tpl (7013)				11968		
Tpl (6999)			15320			
Tpl (6998)		13400				5560
Tpl (7065)	12500(4281)			2530	1560	

a The PBM from this animal were stimulated once *in vitro*, with cells infected with the same *T. parva* parasite stock as was used for immunization, or with another cell line believed to be antigenically different from the immunizing strain, and tested against the panel of target cell lines.

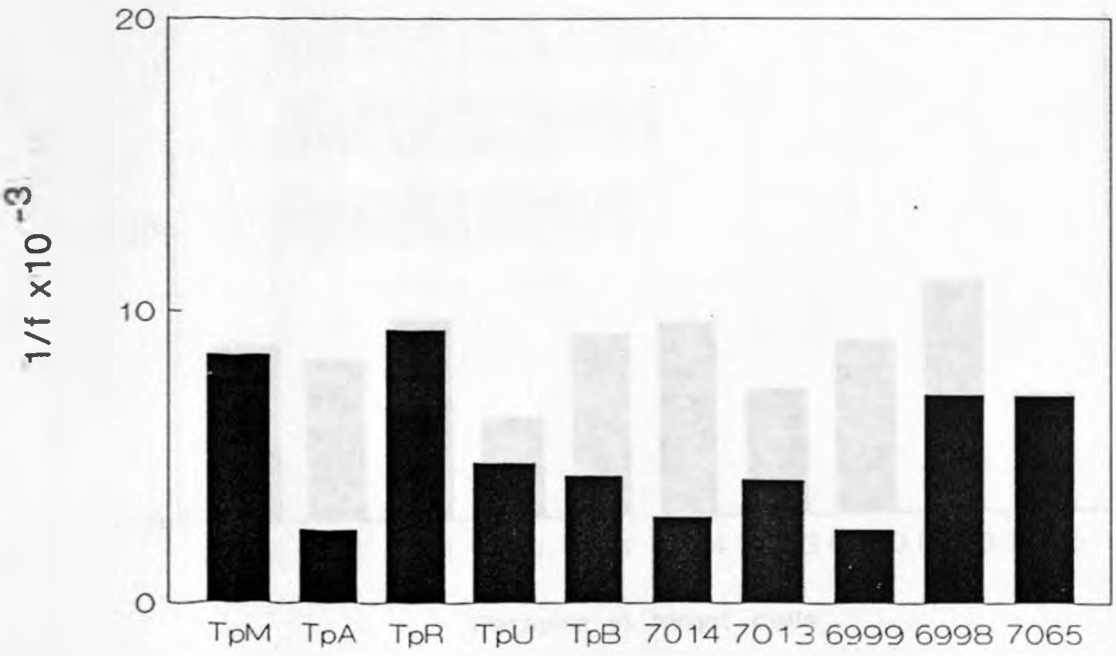
b The reciprocal of the precursor frequency of cytotoxic T-cells. The value in parenthesis is the standard deviation where more than one result was statistically valid.

Fig. 18 Summary of the estimation of the frequencies of cytotoxic T-cell precursors by LDA in the PBM of 5 immune steers; D482 immunized with Tpl (7014), D504 immunized with Tpl (6998), D508 immunized with Tpl (7065), D503 immunized with Tpp (Uganda) and D487 immunized with Tpb (Boleni). PBM were assayed for specific cytolysis on a panel of T.parva-infected cells in an ^{111}In-release cytotoxicity assay. The reciprocal of the frequency (1/f) is plotted. The target cells are indicated as; TpM is Tpp(Muguga),TpA is Tpp(Marikebuni),TpR is Tpp(Mariakani),TpU is Tpp(Uganda) and TpB is Tpb(Boleni).

D482

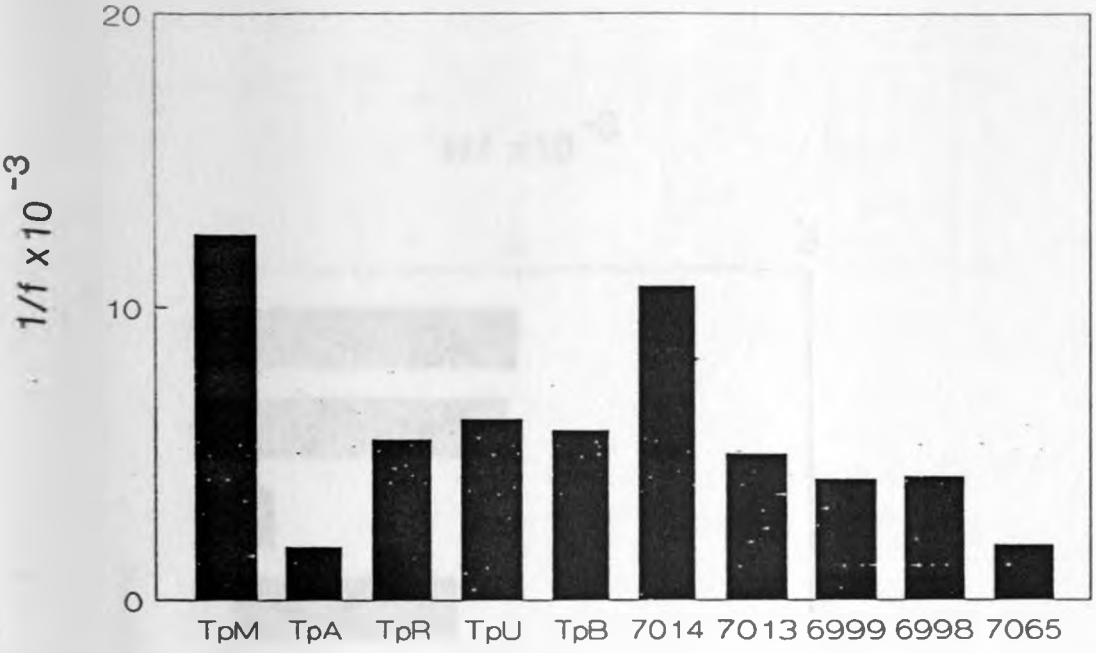


D504

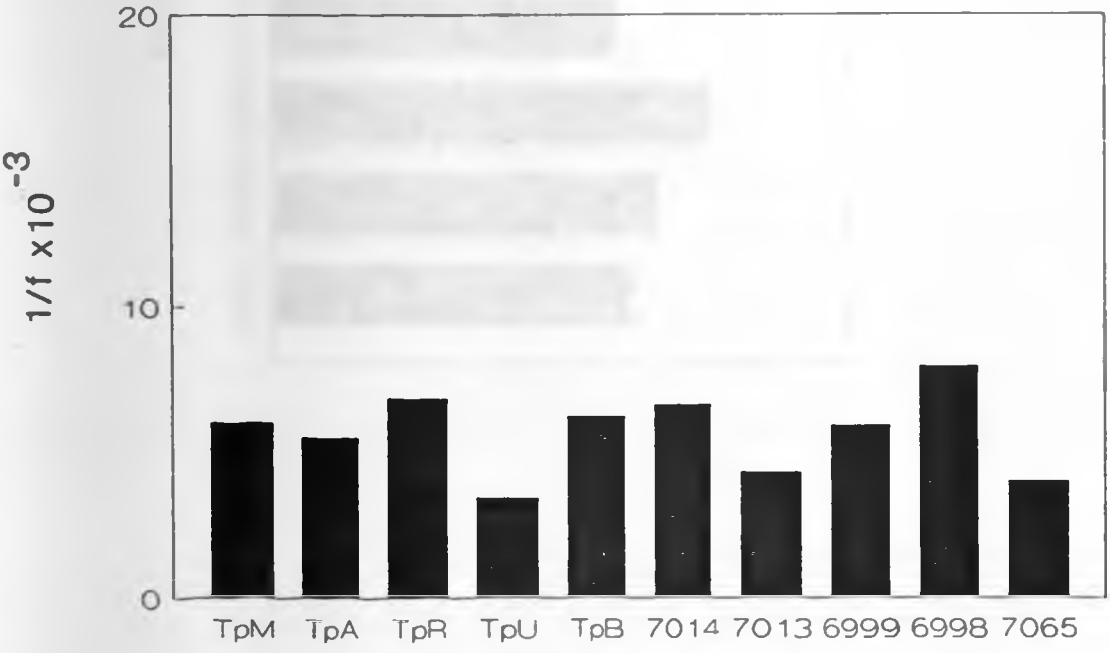


Parasite in target cells

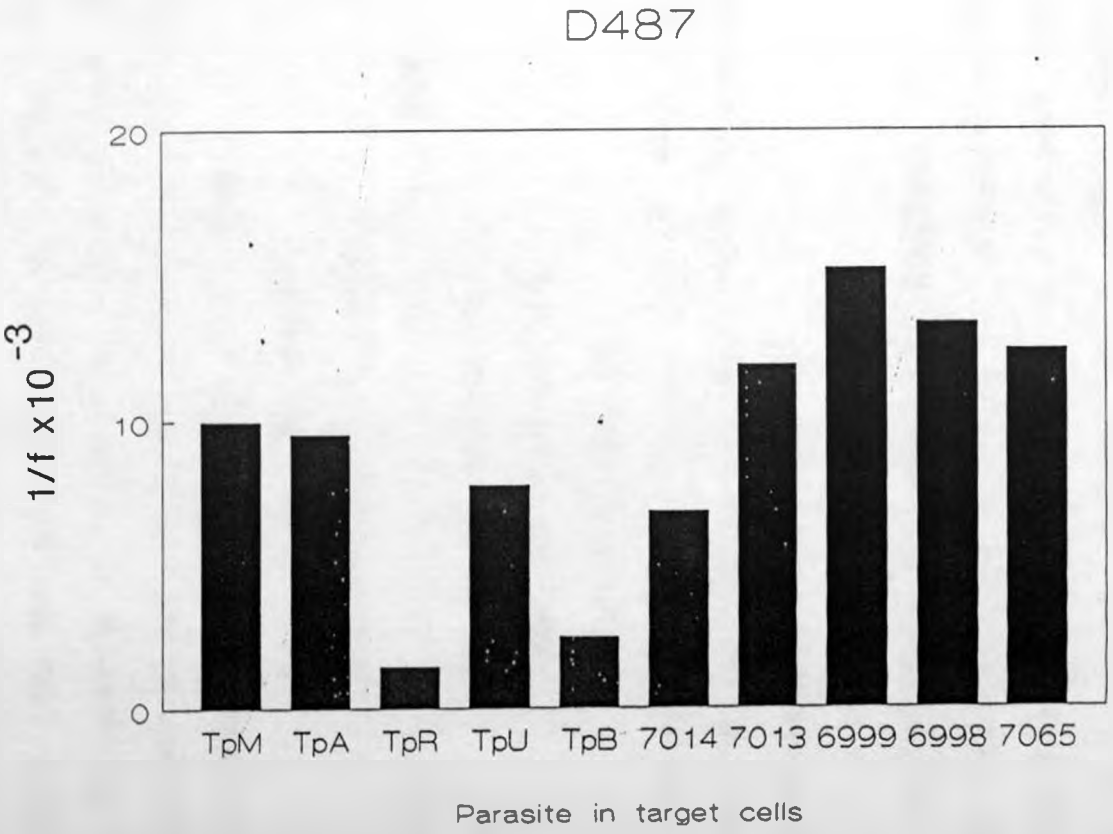
D508



D503



Parasite in target cells


D487
20
10
0
1/f x10 -3
TpM
TpA
TpR
TpU
TpB
7014
7013
6999
6998
7065
Parasite in target cells

3.3.2 SENSITIVITY OF TARGETS TO LYSIS IN THE LIMITING DILUTION ASSAY

The sensitivity of T.parva-infected cell lines to lysis was analysed from the data obtained from limiting dilution assays. The question of interest was whether the other target cell lines were as sensitive as the stimulator line to lysis by cytotoxic T-cells. This was calculated as follows:

$$\text{Sensitivity of target} = \frac{\text{Total no. of wells with killing on the other cell line}}{\text{No. of wells with killing on other cell line which also killed stimulator line}} \times 100\%$$

If the number is greater than 100%, it means that the sensitivity of the "other" cell line to lysis is greater than that of the stimulator line (Table 5).

3.3.3 SPECIFICITY OF CYTOTOXIC T-CELL PRECURSORS

The specificity of the cytotoxic precursor cells was calculated from the LDA data. Since the wells were split and assayed on 2 or more different T.parva-infected target cell lines each time, the analysis of wells with precursor cytotoxic T-cells specific for a T.parva-infected target cell line (which was also used as the stimulator cell line) could be carried out. This was done by considering the number of wells with killing on the stimulator cell line which also killed the other target cell line tested.

Table 5 Sensitivity of target cell line to lysis and specificity of precursor CTL for the stimulator cell line in LDA.

	Stimulator cell line									
	Tpl 7065		Tpl 6998		Tpb (Boleni)		Tpl 7014		Tpp (Uganda)	
Target cell line	SN	SP	SN	SP	SN	SP	SN	SP	SN	SP
Tpl 7065										
Tpl 6998	113%	75							145%	55%
Tpl 7014					116%	68%				
Tpl 7013							110%	93%		
Tpl 6999			227%	69%						
Tpb (Boleni)			118	90%						
Tpp (Uganda)	88%	56%	166%	95%						
Tpp (Marikebuni)							108%	69%		
Tpp (Mariakani)							89%	40%		
Tpp (Muguga)					122%	74%				

SN is sensitivity of test cell line (other than stimulator line) to lysis

SP is specificity of CTL for the stimulator cell line

This is calculated as:-

$$\text{Specificity of cytotoxic T cell precursors} = \frac{\text{No. of wells with killing on stimulator line which also killed 'other' cell line}}{\text{Total number of wells with killing on stimulator line.}} \times 100\%$$

The results for different target cell lines are shown (Table 5).

3.4 CLONED EFFECTOR POPULATIONS TESTED FOR PARASITE SPECIFICITY

Cells from restimulated bulk cultures of PBM from the immune steer D482, which had high cytotoxic activity against the majority of targets infected with buffalo or cattle-derived T.parva parasites, were cloned. Cloning was neccessary to segregate the various effectors present in the potentially heterogeneous population of cells of the bulk culture. Clones allow for assay of a homogeneous effector population to determine if the effector cells recognize an antigen common to all the target cell lines tested resulting in lysis of these cells. Clones were derived from D482 by the procedures outlined in section 2.5 and were assayed for cytotoxic activity on the panel of T.parva-infected cells.

The results indicated that clones D482.32 and D482.38 were able to kill all 10 infected target cell lines tested, including Tpp (Muguga)-infected cells, but did not kill

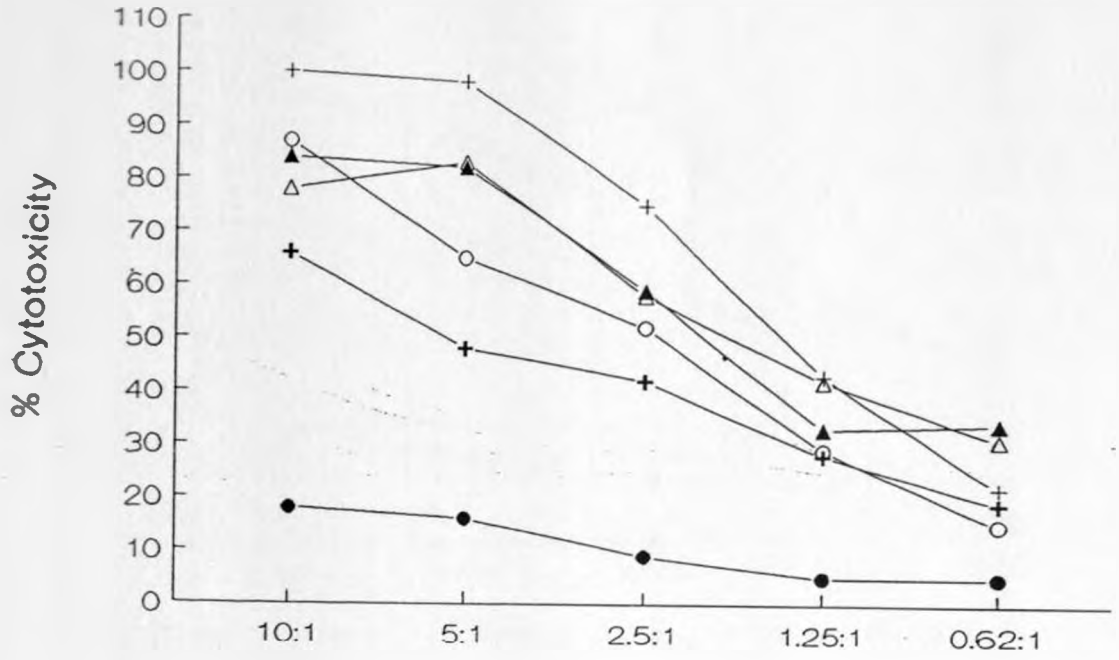
autologous uninfected blasts (Figs. 19 and 20). However the lowest killing was on Tpp (Muguga), (Mariakani) and Tpb (Boleni)-infected cells. The level of cytotoxicity was approximately 50% at an effector to target ratio of 2.5:1 on all 10 infected targets but clone D482.38 gave a lower killing at this E:T ratio for cells infected with Tpb (Boleni) parasite.

Cytotoxicity by the other 2 clones generated, D482.35 and D482.39, was 100% at an E:T of 5:1 for both of the target cells tested which were infected with the buffalo-derived parasites Tpl (7014) and (7013), whereas it was less than 60% on the Tpp (Muguga) and (Marikebuni)-infected cell lines tested (Fig. 21 and 22). The pattern of cytotoxicity on the 4 targets tested is identical for these two clones and most similar to that of clone D482.38. The pattern of cytotoxicity of these 3 clones is distinguished from that of clones D482.32, in that by comparison the killing by D482.32 is higher on Tpp (Marikebuni)-infected target cells and lower on the Tpl (7013)-infected target cells.

Fig. 19 Analysis of the parasite strain specificity of Theileria-specific cytotoxic clone D482.32 from immune steer D482 stimulated with Tpl (7014) and tested on autologous targets infected with different buffalo or cattle-derived T.parva parasites: Tpl (7014) (light crosses),Tpp (Uganda) (open circle),Tpp (Marikebuni) (closed triangle), Tpl (7013) (open triangle),Tpp (Muguga) (bold crosses) and uninfected blasts (closed circle) (Fig.19a),Tpl (7065) (open square), Tpl (6999) (open inverted triangle), Tpl (6998)(open diamond), Tpp (Mariakani) (closed diamond) and Tpb (Boleni) (closed square)(Fig 19b). They were tested in an ^{111}In-release cytotoxicity assay at an E:T ratio of 10:1 or lower.

Clone D482.32

(a)



(b)

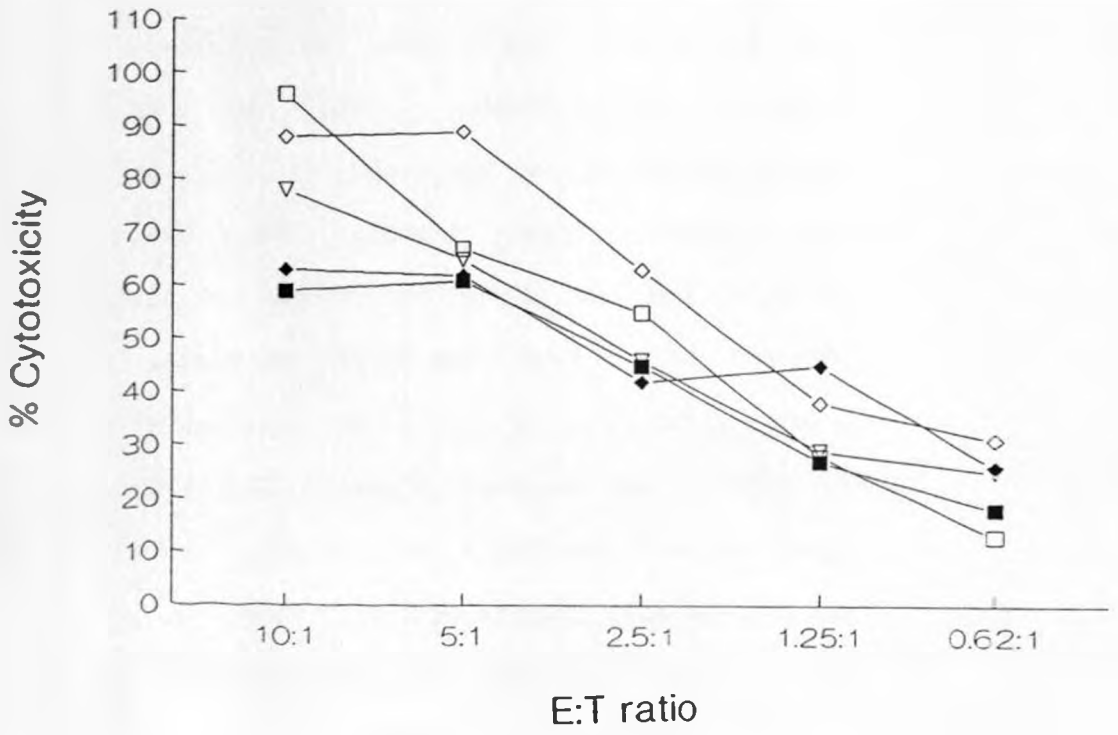
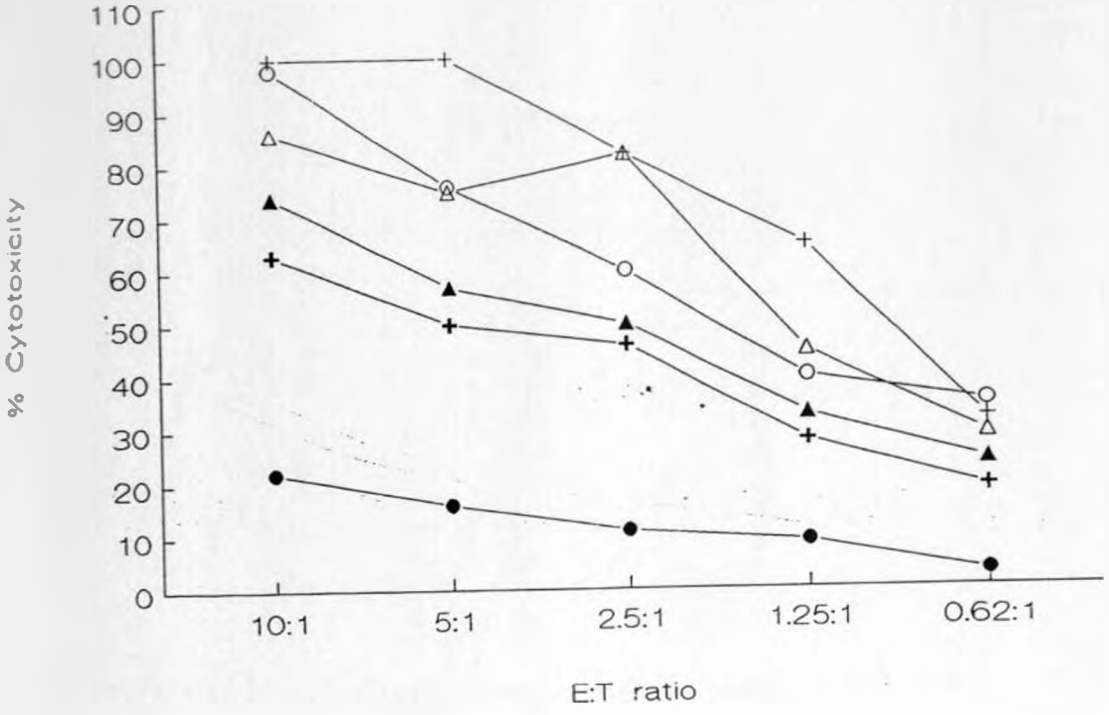


Fig. 20 Analysis of parasite strain specificity of Theileria-specific cytotoxic clone D482.38 from immune steer D482 stimulated with cells infected with Tpl (7014) and tested on autologous targets infected with different buffalo or cattle-derived T.parva parasites, (a) Tpl (7014) (light crosses), Tpp (Uganda) (open circle), Tpl (7013) (open triangle),Tpp (Marikebuni) (closed triangle), Tpp (Muguga) (bold crosses) and uninfected blasts (closed circle), or (b) Tpl (7065) (open square), Tpl (6999) (open inverted triangle), Tpl (6998) (open diamond) Tpp (Mariakani) (closed diamond) and Tpb (Boleni) (closed square). They were tested in an ^{111}In release cytotoxicity assay at an E:T ratio of 10:1 or lower.

Clone D482.38

(a)



(b)

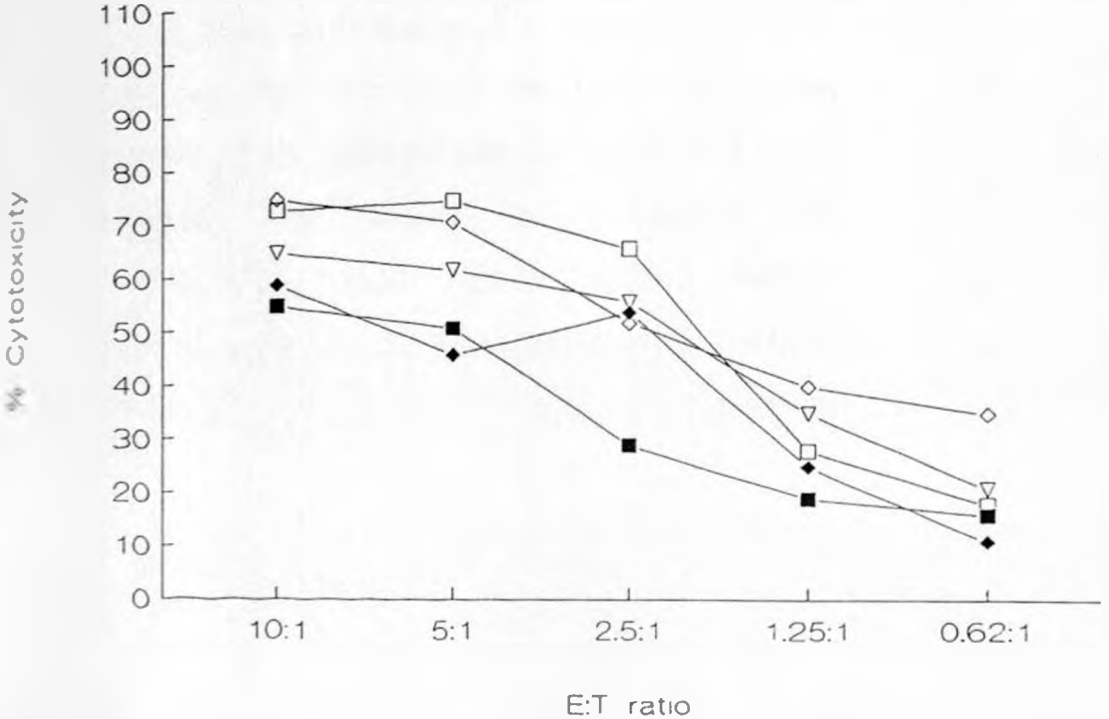
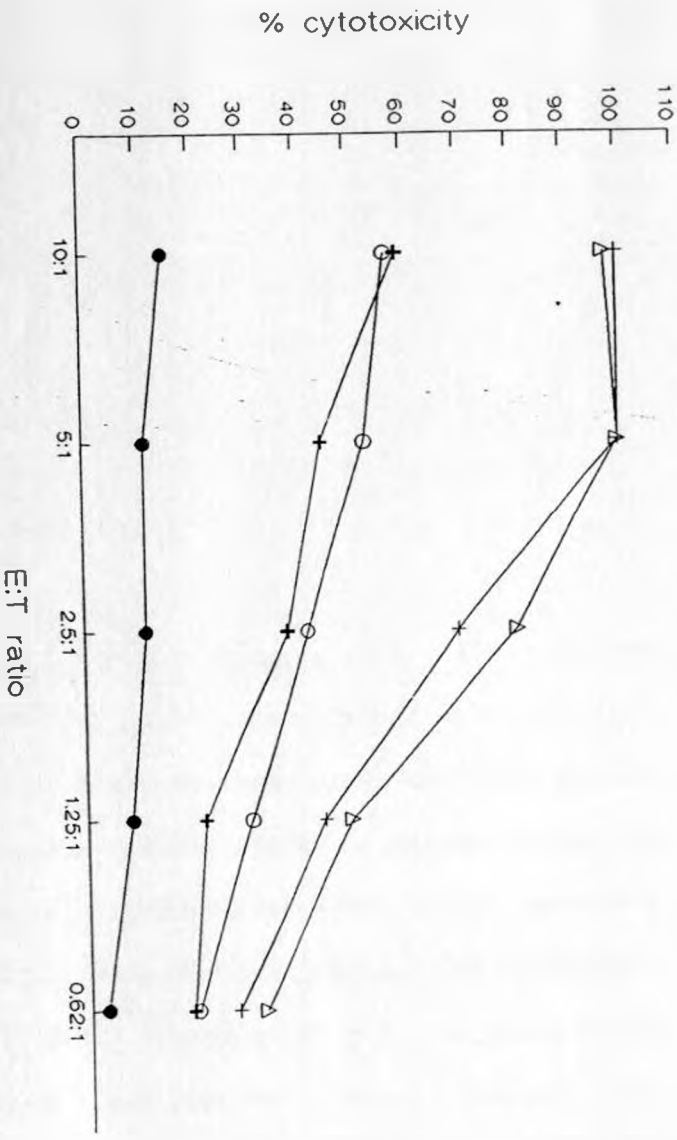
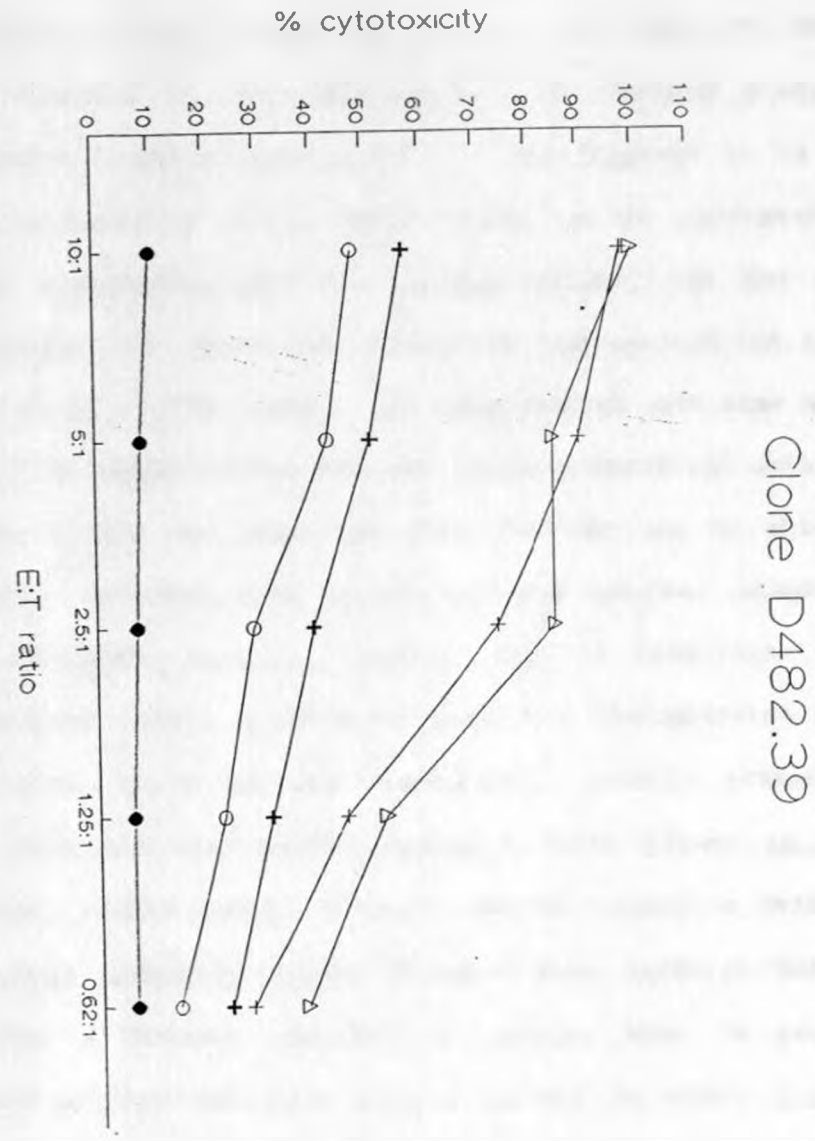


Fig. 21 Analysis of the parasite strain specificity of *Theileria*-specific cytotoxic clone D482.35 from immune steer D482 stimulated with Tpl (7014) and tested on autologous targets infected with different buffalo or cattle-derived *T.parva* parasites: Tpl (7014) (light crosses), Tpl (7013) (open triangle), Tpp (Marikebuni) (bold crosses), Tpp (Muguga) (open circles) and uninfected blasts (closed circles). They were tested in an ^{111}In release cytotoxicity assay at an E:T ratio of 10:1 or lower.



Clone D482.35

Fig. 22 Analysis of the parasite strain specificity of *Theileria*-specific cytotoxic clone D482.39 from immune steer D482 stimulated with Tpl (7014) and tested on autologous targets infected with different buffalo or cattle-derived *T.parva* parasites: Tpl (7014) (light crosses), Tpl (7013) (open triangle), Tpp (Marikebuni) (bold crosses), Tpp (Muguga) (open circles) and uninfected blasts (closed circles). They were tested by an ^{111}In-release cytotoxicity assay at an E:T ratio of 10:1 or lower.


% cytotoxicity
0
10
20
30
40
50
60
70
80
90
100
110
10:1
5:1
2.5:1
1.25:1
0.62:1
E:T ratio
Clone D482.39

CHAPTER 4. DISCUSSION

Immunity to ECF is partially due to a cell-mediated immune response directed at cells infected with the schizont stage of the parasite (Pearson *et al.*, 1979). The response is in the form of cytotoxic T cells (CTL), which can be generated *in vitro* by stimulating PBM from immune animals, but not from naive animals, with their own autologous *T.parva*-infected cells (Pearson *et al.*, 1979, 1982). *In vitro* studies have also shown that CTL from immune cattle can kill cells parasitized with the stock the animal was immunized with, but may not be able to kill cells infected with parasites from another stock of *T.parva* (Morrison *et al.*, 1986b). This is consistent with cross-immunity trials which have shown that immunization with one parasite stock may not necessarily provide protection against infection with another parasite stock (Young *et al.*, 1973, 1975; Radley *et.al.*, 1975a). However there is evidence that *T.parva lawrencei* stocks obtained from infected buffalo may induce a broader immunity in cattle, that is provide protection against challenge with a variety of other *T.parva* stocks (Young *et al.*, 1973; Dolan *et al.*, 1980). Other studies indicate that buffalo are largely resistant to lethal infections by *T.parva* (Barnett and Brocklesby, 1966). This may be partly attributable to the induction of CTL which are able to kill cells infected with a variety of other *T.parva* parasites (Baldwin *et al.*, 1988).

In this study, the parasite strain specificity of CTL from cattle immunized with 1 of 5 different stocks of buffalo-derived T.p.lawrencei parasites was evaluated and compared with CTL of cattle immunized with the cattle-derived T.parva parasite stocks T.p.parva (Uganda) and T.p.bovis (Boleni). The objective was to determine whether any of these parasite stocks was able to generate non-strain restricted CTL that recognize and subsequently lyse cells infected with a variety of buffalo-derived and cattle-derived T.parva parasites. Immune T cells capable of this are postulated to be recognizing parasite-induced or derived antigens on the membranes of infected cells which are common among cells infected with the different T.parva parasite strains. Such an antigen could provide the basis for a vaccine against ECF.

The five buffalo which served as the source of the T.p.lawrencei stocks used for immunization and in vitro infection of lymphocytes were from 2 different localities in Kenya and were therefore likely to represent different strains of T.parva parasites. Characterization by anti-schizont MAbs indicated that there were differences among some parasite stocks although the antigenic profiles between stocks was identical. Different MAb profiles probably mean that the lympocytes were infected with different T.parva parasites but identical profiles do not neccesarily mean that the lympocytes were infected with identical parasites since the detection of schizont membrane antigens by MAbs is unlikely to be comprehensive. The fact that there are different profiles for

cells from different animals infected with the same parasite stock probably indicates that the stocks are composed of more than 1 parasite strain. The 2 cattle-derived parasite stocks, Tpp (Uganda) and Tpb (Boleni), were used for comparative immunization because they had been shown by field observations to provide a wide degree of protection *in vivo* (S. Morzaria, pers. comm.).

The seven experimental animals used for immunization were raised by embryo transfer from a single bull and cow. Three had the MHC phenotype w6/KN8 and 4 had the phenotype w6/KN12. Although they are not genetically identical they have a high level of relatedness. Differences in their immune responses to *T.parva* parasites can therefore probably be attributed to the antigenicity of the parasite rather than to the influence of genetic differences. To further minimize experimental differences due to trivial effects, PBM from 2 of these cattle (one w6/KN8 and one w6/KN12 phenotype) were used for *in vitro* infection with sporozoites to establish the target and stimulator cell lines used in all the assays.

Restimulated bulk cultures of PBM were chosen for evaluating the parasite stock specificity of *T.parva*-specific CTL because they have certain advantages. First, in contrast to *T.parva*-specific CTL generated *in vivo*, which are detected in the blood only for a few days after immunization or challenge of immune animals (Emery *et al.*, 1981; Eugui and Emery, 1981; Morrison *et al.*, 1986a), the capacity to generate CTL *in vitro* weeks or months after immunization permits

repeated access to the same animal. This enables in depth analyses of the immune response to be carried out. In addition, weekly restimulation of cultures from immune animals results in an increase in the frequency of the CTL enabling higher levels of cytotoxicity to be attained, thus providing greater sensitivity in experiments directed at analyzing the specificity of the response (Goddeeris *et al.*, 1986a; Morrison *et al.*, 1986a). Repeated stimulation also reduces the nonspecific killing which is often observed after a single stimulation of PBM *in vitro* (Morrison *et al.*, 1986a). However repeatedly stimulated bulk-cultured cells have the disadvantage that some cells with particular antigen specificities may out grow others thereby becoming the dominant effector population evaluated.

Limiting dilution assays were also used to analyze the antigen specificity of the CTL. Limiting dilution analysis is a standard tool for estimating frequencies of precursors of cytotoxic T-cells (Lefkovits and Waldman, 1984) and provides an approximation of the clonal basis of the immune response. Cytolysis of the target cell identifies the presence of the cytotoxic clone and retrospectively, the parent or precursor cell. Thus cytotoxicity assays were used to measure the number of such clones specific for cells infected with *T.parva* parasites thereby providing information on the number of T-cell precursors which were triggered to respond and from this their frequency in PBM was estimated. It is assummed in such assays that a single CTL specific for *T.parva* parasites, coming into

contact with a T.parva-parasitized cell inevitably sets into motion a chain of events that eventually leads to the lysis of the infected cell.

In addition CTL were cloned from the restimulated bulk-cultured PBM of one of the cattle. These cloned cells have the advantage over bulk-cultured PBM and the results obtained in the LDA in that they represent an effector cell population with a single antigen specificity. If one is interested in identifying an identical antigen or cross-reactive antigens which are common among cell lines infected with a variety of different parasite strains, cloned T cells provide a tool for determining if such an antigen exists. It is assumed that all the target cells killed by a population of cloned cells are bearing a common or cross-reactive parasite-derived or parasite-induced antigen.

Using the procedures outlined in this work and elsewhere (Goddeeris et al., 1986a and 1986b; Morrison et al., 1987a and 1987b), it was possible to generate cytotoxic T-cells specific for cells infected with the protozoan parasite T.parva from all 7 cattle immunized with different stocks of buffalo-derived or cattle-derived T.parva parasites. The cytotoxic activity of the T.parva-specific CTL has been shown to reside in the $BoT4^-/BoT8^+$ population (Goddeeris et al., 1986b). Cytotoxic cells detected here were shown to be restricted by BoLA A-locus-encoded class I MHC antigens as has previously been shown by Goddeeris et al. (1986a) for $BoT8^+$ cells. It was shown that the majority of cytolytic activity of bulk-cultured

PBM, from the 4 animals tested, which had been stimulated 2 to 4 times *in vitro* by *T.parva*-infected cells, was restricted to one of the BoLA haplotypes. A similar bias in restriction of the cytotoxic T cell response to one BoLA haplotype has also been observed *in vivo* and among the BoLA-A locus specificities studied there seems to be a hierarchy in dominance in restriction of response (Morrison *et al.*, 1986b).

Cytotoxic cells generated from animals that had been immunized with the 5 buffalo-derived parasites exhibited remarkable similarity in their parasite strain specificity. There was no killing above 15% on targets of uninfected blasts by CTL from any of the 5 cattle, showing that the effectors were specific for *T.parva*-parasitized cells. The killing on target cells infected with buffalo-derived parasites, by cultures of PBM from all 5 animals usually exceeded 50% but was lower for target cells infected with cattle-derived parasites except for the Tpp (Marikebuni)-infected target cells. Killing on these was comparable to that on cells infected with buffalo-derived parasites, suggesting an antigenic similarity or commonness between cells infected with Tpp (Marikebuni) and those infected with the buffalo-derived *T.p.lawrencei* parasites.

Cytotoxic cells from the steer immunized with Tpp (Uganda) gave greater than 50% cytotoxicity on the target cells infected with the cattle-derived *T.parva* parasites and on 3 of the cell lines infected with *T.p.lawrencei* parasites. Cytotoxic cells from the Tpp (Uganda)-immunized animal also gave high cytolytic activity on Tpp (Muguga)-infected cells and would perhaps be

placed above the animals immunized with buffalo-derived parasites in terms of the broadness of killing by its CTL, since CTL from the later cattle all had low-level killing on Tpp (Muguga)-infected cells.

In contrast, CTL from the Tpb (Boleni)-immunized animal tended to be restricted in their killing to the cells infected with the immunizing strain and those infected with other cattle-derived parasites and therefore based on these results would not be selected as a good candidate for inducing immunity against a large variety of T.parva stocks, as had been previously suggested based on in vivo field observations. More recent cross-immunity trials have further shown that immunization with Tpb (Boleni) provides protection against challenge with itself (Tpb (Boleni), Tpp (Muguga), Tpp (Marikebuni) and to T.p.lawrencei stocks. One of the T.p.lawrencei stocks was from Ngong (Ngong 1) and the second from a buffalo from Nairobi (Buffalo no. 6792) (Irvin et al., in press). This suggests that perhaps the results of the in vitro cytotoxicity assays are not predictive of protection in vivo. It cannot be completely ruled out that the results obtained were not due to the selection of a single antigenic population of CTL in the bulk-cultured PBM, although the LDA results which do not select for a single effector population also indicated that whilst the CTL were good at killing cells infected with Tpb (Boleni) and Tpp (Mariakani) stocks they were poor when tested against the other infected target cells. One cannot rule out the possibility that the particular infected

stimulator cells used do not bias the response. Since the Tpb (Boleni) stock may contain a mixture of parasites it is possible that cells infected in vitro with that stock may be heterogeneous for the parasite-derived antigens or induced antigens expressed on their membranes. In this instance an infected cell population which is not very efficient at stimulating effector cells which recognize common antigens may have been selected. In support of this possibile explanation, when cell lines infected with buffalo-derived stocks were used in LDA as stimulators of PBM from the Boleni-immunized animal the frequency of cytotoxic cells able to kill some of the target cell lines was increased.

An obstacle in the interpretation of results generated using uncloned effectors is that the response detected may be a combined result of having different antigen specificities. Since the experimental cattle were immunized with parasite stocks which may contain several strains or antigenic populations, it is important to define whether the broad killing observed is due to the sum effect of different effector cells recognizing different antigen epitopes or whether this response is due to the recognition of an antigen epitope which is identical or cross-reactive and common among cells infected with the different strains of T.parva. If, when PBM from an immune animal are stimulated with parasites other than the immunizing strain, the pattern of parasite specificity of the effectors is changed, that is increasing or decreasing the cytotoxic activity on some target cell lines, it indicates that

effectors representing more than one antigen specificity are present in the PBM. Presumably the effectors predominating in the second instance would be recognizing antigens dominant on that stimulator cell line whereas the immunizing strain-stimulated cultures would predominantly be recognizing a different antigen epitope, probably the immunodominant one on cells infected with that strain of parasite.

To investigate this, cultures of D482 PBM were stimulated with cells infected with the immunizing strain Tp1 (7014) or with Tpp (Muguga)-infected cells. The Tpp (Muguga)-infected cells had been shown to have a stimulatory capacity comparable to the Tp1 (7014)-infected cells in proliferation assays but were not highly killed by D482 effectors generated in bulk cultures stimulated with Tp1 (7014)-infected cells. Effectors raised against Tp1 (7014)-infected cells gave high killing on cells infected with the immunizing/stimulating strain and on cells infected with other buffalo-derived parasites. The killing on Tpp (Muguga)-infected cells was however low. Stimulating with Tpp (Muguga)-infected cells and testing on target cells infected with Tpp (Muguga) and other cells infected with cattle-derived parasites, gave high killing on all of them but not on cells infected with buffalo-derived parasites such as Tp1 (7014). Thus it is possible to stimulate effectors which have different antigen specificities depending on the stimulator cell line used. A similar experiment, done with PBM from D482 and tested in the LDA after stimulation with either Tp1 (7014) or Tpp (Muguga)-infected cells, gave the same

result regarding Tpp (Muguga)-infected target cells, further supporting the contention that at least 2 different Theileria-specific effector populations occur in the PBM of D482.

Similarly, if the stimulator cell line is a bulk culture of uncloned cells it may consist of more than one population of infected lymphocytes which bear distinct antigens on their surface. The various populations may preferentially stimulate effector cells with different antigen specificities. These different effectors would potentially yield different levels of killing on target cells infected with different parasite stocks, depending on which antigen predominated on the surface of the infected target cells. The levels of killing on different target cells would therefore vary depending upon which type of effector cell predominated in the bulk culture.

To address this possibility, PBM from animal D482 Tp1 (7014)-immunized were stimulated with a clone of the Tp1 (7014)-infected cell line. The specificity of the killing changed in that although the killing on some targets resembled that obtained with cultures generated by stimulating PBM with the uncloned infected cell line, there were significant differences. The killing on the uncloned Tp1 (7014)-infected cell line decreased when the cytotoxic cells were generated by stimulating cultures with the cloned Tp1 (7014)-infected cell line. This indicates that the effectors generated by stimulating with a cloned-infected cell line either did not react with as many cells within the bulk (uncloned) Tp1

(7014)-infected cell line or they recognized antigens which were expressed either at low levels on the cell surfaces or they recognized antigens for which they had a lower affinity. This indicates that the killing of target cells infected with buffalo-derived T.p.lawrencei parasites, and some infected with cattle-derived T.p.parva parasites, by bulk-cultured PBM from D482, is probably either dependent on stimulation with a mixed population, resulting in generation of effector cells with specificities for more than one parasite-induced antigen epitope or dependent on stimulation with an infected cell population not represented by the cloned Tp1 (7014)-infected cell line.

The results for the 5 immune animals tested in LDA generally reflected the pattern obtained with bulk cultures. In some instances the pattern of results in the LDA and bulk cultures differed. For example, D508 immunized with Tp1 (7065) had a low frequency of effectors specific for Tp1 (7014)-infected cells in the LDA, while the bulk culture gave high cytolytic activity. The frequency of CTL specific for cells infected with the immunizing strain was generally higher than that for cells infected with different parasites. However, when cultures in the LDA were stimulated with cells infected with parasites other than the immunizing strain, the specificity sometimes changed in that the highest frequency obtained was for effectors which killed the stimulator cell line, even though it was infected with a different stock of parasite than that used to immunize the animal. The similarity

of results between the restimulated bulk cultures and the LDA results indicate that although the bulk cultures most likely consisted of mixed effector populations which had the opportunity to be disproportionately represented after repeated stimulations *in vitro*, as compared to their relative proportions found in the lymphoid system of the immune cattle, this disproportionality probably did not occur to a great degree.

Although the LDA provides an estimation of the proportion of cells in the PBM which are able to kill cells infected with the various parasite stocks it, like the bulk cultures, does not allow us to determine if the same antigen is being recognized on all of the different infected target cell lines. Therefore, to see if the cytotoxicity measured against the targets infected with a variety of different stocks could be accounted for by the expression of a common or cross-reactive parasite-derived or induced antigen, effector cells were cloned from the restimulated bulk cultures of animal D482, Tp1 (7014)-immunized. The 4 clones generated gave significant cytotoxic activity on all the target cells infected with buffalo- or cattle-derived *T.parva* parasites, indicating that they were recognizing an antigen expressed on all the *T.parva*-infected cell lines tested. All 4 clones had high cytolytic activity (greater than 90%) on *T.p.lawrencei*-infected cells but lower, although significant, killing on Tpp (Muguga)-infected cells (less than 60% at E:T ratio of 5:1). To a considerable extent the results with the clones support

the cytotoxicity pattern of the bulk-cultured cells in that the lowest killing was on cells infected with Tpp (Uganda), (Mariakani) and Tpb (Boleni). This means that the Tpl (7014)-stimulated bulk cultures may have been predominantly comprised of a single effector cell type. It would seem however that the clones may be recognizing 2 different common parasite-derived or induced antigens on the surface of infected cells, since their patterns of cytotoxocity differed. However both antigen epitopes seem to be expressed on all the cell lines, but the relative quantities varies among cell lines infected with different stocks.

The experiments with restimulated bulk-cultured T cells, the limiting dilution assays and cloned T cells indicate that antigens common or cross-reactive among cell lines infected with different parasite stocks may occur fairly frequently. The experiments with the cloned CTL suggest that probably at least two different common or cross-reactive antigen epitopes are expressed on all the cell lines infected with the different parasite stocks tested, although their expression may vary among cells infected with different parasite strains, resulting in the partial killing of some target cell lines by cloned effectors. This could be explained by various mechanisms. Depending on the cell cycle, the relevant antigen may or may not be expressed on the surface of all the parasitized cells at the same time. Secondly, the common or cross-reactive antigenic determinants may be masked by strain specific antigens or only expressed at low levels on cells infected with

some of the strains of T.parva leading to low affinity binding of target cells by cloned cytotoxic effectors. In addition the antigen epitope may not be identical for all cell lines infected with different parasite strains but rather be cross-reactive on some of the parasitized cells. A cross-reactive antigen would logically have lower affinity for the T-cell receptor of the cloned effector cells. The partial killing of some of the infected cell lines is probably not indicative of an inability of such CTL to inhibit growth of these cells. Morrison et al. (1987b) have previously shown that cloned effectors which give partial killing of Tpp (Marikebuni)-infected cells are able to markedly inhibit growth of these same cells in vitro.

The objectives of this study were to determine whether any of the 5 buffalo-derived T.parva parasites is able to generate in cattle non-strain restricted immune T cells that can recognize and subsequently lyse cells infected with a wide variety of buffalo- and cattle-derived T.parva parasites and to determine whether or not such a broad specificity of killing, if it occurs, can be accounted for by the presence of a single antigen epitope on the membrane of infected cells. From the results obtained it would seem that any of the 5 buffalo-derived parasite stocks generates, in animals immunized with them, effector cells which recognize antigen epitopes expressed on a large number, if not all, of the target cells tested. In order to correlate these in vitro observations with the in vivo immune status of each animal the animals should be

challenged with other parasites and their response monitored. Only if such challenge experiments and cross-immunity trials turn out to be succesful would the relevant parasite stock(s) be considered for immunization trials in the field.

Considerable research is in progress to identify the antigenic determinants on schizont-infected cells that are recognized by Theileria-specific cytotoxic T lymphocytes. Such antigens might provide the basis for a recombinant subunit vaccine against this stage of the parasite. An effective vaccine against ECF will probably have to combine more than one of the major parasite-induced or derived antigens which are expressed on the surface of schizont-infected cells. It would be advantageous if they were common among cells infected with a variety of different parasite stocks. The clones generated during the course of this study may provide tools to identify such antigen epitopes and the parasite genes encoding for them.

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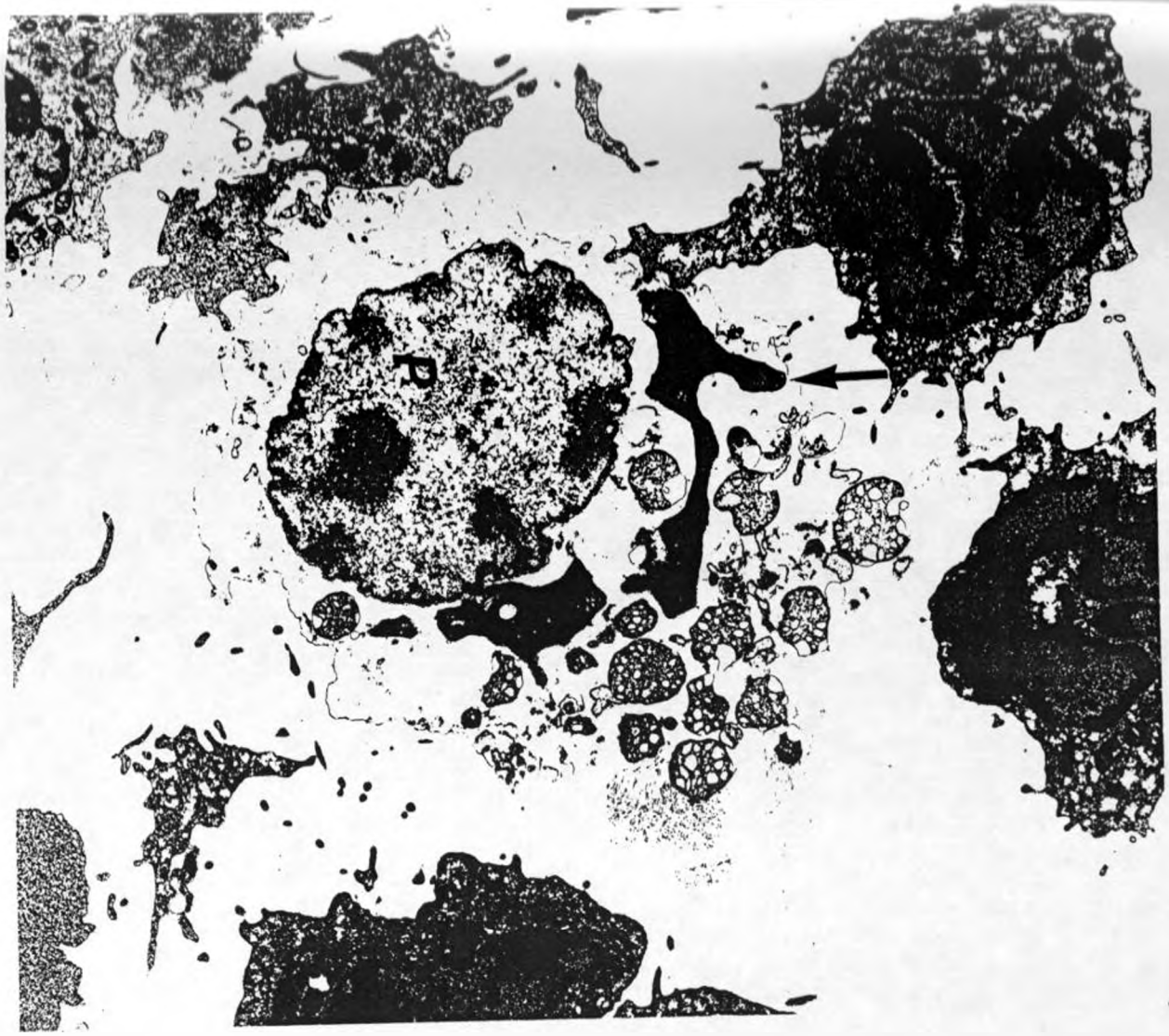
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