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Cell-mediated immune responses to *Theileria parva* (East Coast fever) during immunization and lethal infections in cattle

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Summary. Leucocytes from cattle with lethal or sub-lethal infections with *Theileria parva* were examined for responses in autologous mixed lymphocyte reactions (MLR) and for specific and non-specific cytotoxicity against cultured cell lines. During lethal infections, lymphoblasts isolated from central lymph from days 8 to 14 stimulated proliferation in autologous lymphatic lymphocytes and peripheral blood leucocytes (PBL), although cytotoxicity was not generated in such cultures. After day 14, non-specific cytotoxicity was exhibited by both lymphocyte populations and was maximal in moribund calves. Non-specific lytic activity was also noted in PBL from recovering calves, but disappeared after the elimination of the parasite.

Calves immunized against *T. parva* by the concurrent administration of stabilate and long-acting tetracycline showed macroschizont forms of the parasite in host lymphocytes from days 15 to 18 after infection. During this period, lymph node cells from infected calves stimulated MLR-type responses in autologous PBL. However, PBL from immunized calves lysed directly, autologous infected lymphoblasts in a genetically-restricted fashion from days 14 to 21, although additional cytotoxicity was not generated in the MLR. The results imply that when the parasitosis of *T. parva* is curtailed, specific cell-mediated responses are mounted against parasite-induced antigens in combination with polymorphic host antigens on the leucocyte membrane. These reactions are probably the major immune responses conferring to recovered cattle, immunity against rechallenge with the homologous isolate of *T. parva*.

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INTRODUCTION

Infection of the lymphoid system of susceptible cattle with the protozoan parasite *Theileria parva* is characterized by progressive proliferation and destruction of host lymphocytes which results in the death of cattle 2–4 weeks after challenge. In cattle which recover, resistance to reinfection with the homologous isolate of *T. parva* persists for several years (Burridge, Morzaria, Cunningham & Brown, 1972), although such animals are susceptible to varying degrees to infection with different isolates of *T. parva* (Cunningham, 1977).

Attempts to characterize the protective response(s) of cattle against Theileriosis initially examined the serological responses against the macroschizont and piroplasmic stages of the parasite. Immunization of cattle with purified antigens of *T. parva* (Wagner, Duffus & Burridge, 1974) or inactivated schizonts of *Theileria annulata* (Pipano, Goldman, Samish & Friedhoff, 1977) elicited the production of specific antibody which did not protect cattle against a

subsequent lethal infection. Comparable studies of cell-mediated immunity to *T. parva* in cattle were restricted by the availability of autologous cell lines on which to assay responses; this limitation was surmounted by the ability of sporozoites of *T. parva* to transform *in vitro* normal bovine leucocytes into parasitized lymphoblastoid cell lines (Brown, Stagg, Purnell, Kanhai & Payne, 1973). Recently it has been reported that peripheral blood leucocytes (PBL) from immunized cattle display both direct specific cytotoxicity (Eugui & Emery, 1980) and cytotoxicity *in vitro* in the mixed lymphocyte reaction (MLR) when stimulated by autologous lymphoblasts from cell lines infected with *T. parva* (Pearson, Dolan, Stagg & Lundin, 1979).

Since cell-mediated lympholysis (CML) was a feature of the response to *T. parva* of immune cattle, the present study examined the evolution *in vivo* in PBL and efferent lymphatic lymphocytes (ELL) of proliferative mixed lymphocyte reactions (MLR) and CML reactions during the course of East Coast fever. The findings suggest strongly that cell-mediated immunity is the major means of protection against *T. parva* in cattle, and that a cytotoxic cell response is mounted in primed cattle against parasite-induced antigens in combination with one or more polymorphic host antigens on the leucocyte membrane.

MATERIALS AND METHODS

Animals and infection

Cattle. Thirty Friesian steers aged 5–8 months and weighing approximately 100 kg were housed in insect-proof isolation units during the experimental period and fed concentrate rations. All cattle were screened monthly as serologically negative for the presence of antibodies to *Theileria, Trypanosoma, Babesia* and *Anaplasma* species.

Infection. A lethal dose of *T. parva* (Muguga), ILRAD stabilate IRPOI 001/V was injected subcutaneously (s.c.) in each flank (0·5 ml in each site) to drain to the prefemoral lymph nodes. The origin of the stabilate has been described previously (Morrison, Buscher, Murray, Emery, Masake, Cook & Wells, 1981). The calves died 18–20 days after challenge.

Immunization. Twelve cattle were inoculated with a lethal dose of *T. parva* (Muguga) and concurrently were injected intramuscularly in the neck with a single dose of long-acting tetracycline (Terramycin-LA, Pfizer, Sandwich, Kent, batch 803-53021-1) at a dose of 20 mg/kg body weight. This produces a sub-lethal infection after which the animals are immune (Radley, Brown, Cunningham, Kimber, Musisi, Payne, Purnell, Stagg & Young, 1975).

Surgical procedures

The collection of lymphocytes in central lymph efferent from the prefemoral lymph nodes of cattle was facilitated by the establishment of indwelling lymphatic cannulae (Hall, 1967). Lymphocytes were collected into sterile flasks which contained a final concentration of 10 i.u./ml heparin (Novo Industrie A/S, Copenhagen, Denmark).

Separation of PBL and lymphoblasts from blood and lymph

Peripheral blood leucocytes were isolated from blood containing 10 i.u./ml heparin (Novo Industrie A/S, Denmark) by density centrifugation as described by Emery & Morrison (1980). Leucocytes harvested from Ficoll-paque (Pharmacia, Uppsala, Sweden) were washed twice in RPMI 1640 culture medium (Flow Labs, England) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS, Gibco, N.Y., batch K292101D), 10^{-4}M 2-mercaptoethanol (Sigma Chemical Co. St. Louis, Mo.) 10 mM HEPES buffer, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 20 μg/ml glutamine. Blast and infected lymphocytes were separated from smaller efferent lymphatic lymphocytes by centrifugation on density gradients containing 16% and 17% (v/v) metrizamide (Nyegaard, Oslo, Norway). The solutions were layered consecutively in the order: 2 ml of 17% metrizamide, 2 ml 16% metrizamide and 4 ml medium containing lymphocytes. The gradient was centrifuged at 700 ***g*** for 30 min at 4°. Lymphoblasts comprised 60%–80% of the cells harvested above the 16% metrizamide (fraction 1) but less than 5% of cells in fraction 2 (between 16% and 17% metrizamide). Dead cells and erythrocytes deposited beneath the 17% metrizamide. The lymphocytes from fractions 1 and 2 were washed and resuspended in the same culture medium as were the PBL.

Cell lines

Lymphoblastoid cell lines infected with T. parva. Normal PBL from six calves were transformed *in vitro* after cultivation with sporozoites of *T. parva* (Muguga) using the method described by Brown *et al.*, (1973). Macroschizonts of *T. parva* were observed in

lymphoblasts 6 days after infection *in vitro* and the parasitized cells used for cytotoxicity assays were prepared from the exponential phase of cell growth (1 day after passage) and greater than 90% contained macroschizonts of *T. parva*. Lymphoblasts were identified as greater than 12 μm diameter, and macroschizonts were detected by immunofluorescence as described previously (Emery & Morrison, 1980). The macroschizont index (MSI) was expressed as the percentage of lymphocytes which contained macroschizonts.

Murine tumour cell line. A tumour cell line of xenogeneic origin was used as targets to evaluate spontaneous cell-mediated cytotoxicity (SpCC) in PBL and central lymph lymphocytes of different groups of cattle. In preliminary experiments designed to test SpCC in normal cattle, different xenogeneic tumour cell targets both from mice and humans were evaluated. The YAC-1, an *in vitro* established cell line from a Moloney virus-induced lymphoma in A/Sn mice (Cikes, Friberg & Klein, 1973), was the only one susceptible to lysis by normal PBL of cattle under the test conditions (Eugui, unpublished observations). The cells were maintained in RPMI 1640 culture medium, supplemented with penicillin (100 i.u./ml) streptomycin (50 μg/ml), glutamine (20 μg/ml) and 10% heat-inactivated foetal bovine serum (Gibco, N.Y.).

Mixed lymphocyte reaction

Lymphoblasts and PBL which had been separated from central lymph and blood respectively were treated with mitomycin C, 25 μg/ml for 30 min at 37°. The cells were washed three times in culture medium and were resuspended at $2{\cdot}5 \times 10^6$ cells ml for use as stimulator cells. Normal autologous and allogeneic PBL which had been frozen and reconstituted according to the method described by Holden, Oldham, Ortaldo & Herberman (1976) were adjusted to 10^7 or 5×10^6 PBL ml as responder cells. The MLR was established and cultured for 4 days at 37° in a humidified atmosphere of 5% carbon dioxide in air as previously described (Emery & Morrison, 1980). The stimulation index (SI) was computed as follows

$$\text{stimulation index} = \frac{\text{c.p.m. in test wells}}{\text{c.p.m. of PBL in medium}}. \quad (1)$$

For the generation of cytotoxic lymphocytes in the MLR, 1 ml of stimulator cells from a lymphoblastoid cell line at 10^6 per ml were added to 1 ml of responder PBL (5×10^6 per ml) in each of eight wells of a culture plate twenty-four wells × 16 mm (Linbro Scientific Co., Conn.). The cultures were incubated for 5 days at 37° in an humidified atmosphere of 5% carbon dioxide in air. Viability of the cells harvested after this interval was determined in a haemocytometer by the exclusion of eosin dye.

Cytotoxicity assays

These employed the release of ^{51}chromium over a 4-hr period (microcytotoxicity) as described by Pearson *et al.*, (1979) and over a 12-hr period (macrocytotoxicity) (Eugui & Allison, 1979). For the former assay, 10^7 target cells, which were lymphoblasts either separated from central lymph, or from cell lines infected with *T. parva*, were labelled with 200 μCi ^{51}chromium (Radiochemical Centre, Amersham) in 1 ml RPMI culture medium containing 10% FCS for 1 hr at 37°. The cells were washed three times in a culture medium and were adjusted to a concentration of 10^6 per ml. Effector cells generated either *in vivo* (PBL and ELL) or from the MLR *in vitro* were adjusted to 2×10^7 per ml. For the assay, 200 μl of effector cells were added in duplicate to 50 μl of target cells at effector:target ratios of 80:1, 40:1, 20:1 and 10:1 in flat-bottomed microtitre plates (Linbro IS 96-TC, Conn.). Target cells in medium alone acted as a measure of spontaneous release of isotope. Maximum lysis was effected by incubation of target cells with distilled water (total release). After incubation for 4 hr at 37° in an humidified atmosphere of 5% carbon dioxide in air, the plates were centrifuged at 800 ***g*** for 10 mins and 100 μl of supernatant was removed from each well and counted in a gamma scintillation spectrometer (Packard). For the macrocytotoxicity assay, $5{-}10 \times 10^6$ target cells were labelled by incubation for 30 min at 37° in 0·5 ml RPMI 1640 with 5% FCS and 150 μCi ^{51}Cr (as Na_2 ^{51}Cr O_4, Radiochemical Centre, Amersham). Cells were washed twice and left in suspension for 30 min at room temperature before a third wash. A standard number of 4×10^4 labelled cells was added in plastic tubes, to 1 ml volume of effector lymphocytes whose concentration was adjusted to give a final ratio of 100:1 and 50:1 effector to target, respectively. Each sample was evaluated in triplicate and appropriate controls were included in which only target cells were incubated, to estimate spontaneous release. Maximum release was determined by treating the target cell controls with Triton, which releases above 85% of the total ^{51}Cr. After 12 hr incubation at 37° in an atmosphere of 5% CO_2, tubes were centrifuged 10 min at 500 ***g*** and half of

the supernatant (500 μl) transferred to a 3 ml plastic tube and counted in a gamma scintillation spectrometer. Results from both assays were expressed as the percentage of specific lysis according to the formula

$$\% \text{ Lysis} = \frac{\text{c.p.m. in test} - \text{c.p.m. in spontaneous release control}}{\text{c.p.m. in total release control} - \text{c.p.m. in spontaneous release control}} \quad (2)$$

Experimental design

Lethal infection. Eighteen cattle were each inoculated with *T. parva* (Muguga) from which three recovered and fifteen died (Fig. 1). Before infection, five calves from the latter group were submitted for cannulation of the efferent lymphatics from the prefemoral lymph node draining the site of inoculation of *T. parva*. On days 7–11 and 13–16, lymphocytes were separated into blast and small lymphocyte fractions for testing of MLR reactivity and cytotoxic responses against autologous cells. On each of days 8–11 after challenge, PBL and ELL were cultured for 5 days in an MLR with autologous lymphoblasts isolated from central lymph on the day of establishment of the MLR. Lymphocytes harvested from the MLR, PBL and efferent lymphatic lymphocytes (ELL) were tested as effector cells against autologous lymphoblastoid target cells which were isolated for the cytotoxicity assay from central lymph respectively on days 13, 14, 15 and 16 after challenge.

PBL from all fifteen animals were tested at intervals after challenge for cytotoxicity against an allogeneic lymphoblastoid cell line infected with *T. parva*; PBL from two calves and four calves, respectively were tested against an autologous infected cell line and a xenogeneic tumour cell line as well.

Immunization. PBL from calves immunized with *T. parva* and from calves which recovered from a challenge with stabilate were tested at intervals after infection for cytotoxicity against autologous, allogeneic and xenogeneic cell lines (Fig. 1 and Table 1). PBL from three of these calves were cultured in an MLR as responders against the respective autologous cell line on days 0, 7, 14, 18, 21 and 28 after challenge to examine the evolution of MLR-generated cytotoxic responses. Four calves, two from each group, were cannulated to monitor lymph node responses and parasitosis during immunization or recovery. In five immunized calves, the lymph node draining the site of

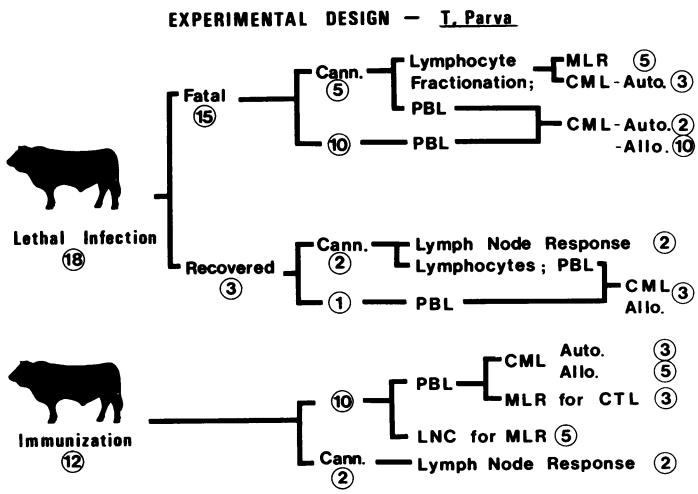

Figure 1. Experimental design for analysis of lymphoid responses during lethal and sub-lethal infections with *T. parva*. Abbreviations include: MLR, mixed lymphocyte reaction; Cann., cannulation of lymphatic ducts; CML, cell-mediated lympholysis; CTL, cytotoxic T lymphocytes; PBL, peripheral blood leucocytes; Auto, autologous cell line; Allo, allogeneic cell line. Numbers of calves studied are encircled.

Table 1. Groups of cattle tested for cell-mediated cytotoxicity during infection with *T. parva*

			Target cells* — Number of cattle examined			
				Infected cell line		
Experimental group	Total number of Animals	Effector cells	Autologous lymphoblasts	Autologous	Allogeneic	Xenogeneic tumour cell line
Lethal infection	15	PBL	5	2	15	4
		ELL	5	ND	ND	ND
		MLR-PBL† (ELL)	5	ND	ND	ND
Spontaneous recovery	3	PBL	ND	ND	3	3
		ELL	ND	ND	2	2
Immunization	7	PBL	ND	3	3	3
		MLR-PBL†	ND	3	3	ND

*Target cells were obtained from the central lymph of infected calves (autologous lymphoblast) or from lymphoblastoid cell lines infected with *T. parva*.

†Effector cells were harvested from 5-day mixed-lymphocyte reactions (MLR) using autologous parasitized cells as stimulators and peripheral blood leucocytes (PBL) or efferent lymphatic lymphocytes (ELL) as responders. (ND, not done).

inoculation of *T. parva* was biopsied at 3-day intervals after infection to provide stimulator cells for a MLR with normal autologous PBL which had been cryopreserved prior to the experiment.

RESULTS

Cell-mediated immune responses during a lethal infection with *T. parva*

Mixed lymphocyte reaction (MLR). The reactivity in the MLR of leucocytes from infected cattle to autologous and allogeneic stimulator cells from peripheral blood and central lymph from the draining lymph node is represented in Table 2. Lymphoblasts isolated from central lymph on days 7–9 after challenge (fraction 1) induced significant proliferative responses ($P < 0{\cdot}05$) in autologous responder cells from lymph but not in autologous PBL. In contrast, lymphoblasts fractionated on days 13 and 14 after challenge initiated MLRs in autologous lymphocytes from all three sources. Over the same period, small lymphocytes from central lymph (fraction 2) failed to stimulate autologous or allogeneic leucocytes to proliferate in the MLR. Peripheral blood leucocytes isolated on days 13 and 14 from infected calves initiated autologous MLRs in small lymphatic lymphocytes, and ELL were stimulatory for the same responder cells on days 9 and 13 after infection. In all instances, the stimulatory capacity for autologous cells of lymphocytes and PBL appeared to parallel the appearance of blast and parasitized lymphocytes in either central lymph or peripheral blood. High levels of incorporation of isotopic nucleotide by control cultures of lymphoblasts (fraction 1) precluded their use as responder cells in the MLR, so that the presence of reactive cells within this sub-population could not be evaluated.

When allogeneic MLR responses were examined on day 9, PBL and lymphoblasts elicited greater proliferation of allogeneic leucocytes than either ELL or small lymphocytes. However, allo-reactive lymphocytes appeared to be represented equally among each population of responder cells.

Cell-mediated lympholysis (CML) of autologous lymphoblasts. The results of the preceding section indicated that infected lymph-borne blast cells initiated MLR-type responses in autologous leucocytes. To examine whether cytotoxicity was developed against these cells during the course of East Coast Fever, ELL or PBL which had been cultured in an MLR with autologous lymphoblasts were tested for cytotoxicity (Table 3). However, only low levels of CML (less than 10% specific lysis) were exhibited against autologous lymphoblasts by effector cells harvested from 5-day MLRs or by ELL or PBL obtained from infected calves on the day of the assay.

Cytotoxicity of peripheral blood leucocytes for autologous, allogeneic and xenogeneic cell lines. For the first 10 days after challenge with *T. parva*, the direct cell-mediated cytotoxicity of PBL from infected cattle for an allogeneic cell line infected with *T. parva* and for a xenogeneic tumour cell line (YAC-1) was similar to that effected by PBL from normal calves (Fig. 2). The specific ^{51}chromium release from autologous target cells during this period was consistently less than 2% and 15% for 4-hr and 12-hr assays, respectively. After day 12, spontaneous non-specific cytotoxicity of PBL for allogeneic infected cell lines and YAC-1 cells increased to reach maximum lytic activity of 18%–40% on day 18. In the corresponding assays, maximum lysis was achieved at effector: target cell ratios of 40:1 (4 hr) and 50:1 (12 hr)—Fig. 2. The lysis of allogeneic cell lines was also effected by thoracic duct leucocytes (TDL) and by LNC biopsied from the draining lymph node 18 days after challenge (data not shown). Responses declined slightly in moribund calves around day 20. In contrast, two calves which succumbed to the infection failed to exhibit in their PBL specific cytotoxicity for autologous infected cell lines at any stage during the disease (Fig. 2).

Responses by calves which recovered from a lethal dose of *T. parva*

Kinetics of the response in central lymph. The output of normal and infected lymphocytes in efferent lymph from the lymph node draining the site of inoculation of *T. parva* in two calves which recovered naturally from the infection is shown in Fig. 3. For the first 4 days after challenge the total output of lymphocytes, blast cells and parasitized lymphoblasts was identical to that induced by a lethal dose of *T. parva* (Emery 1981, submitted for publication). Following the challenge, the lymphocytic output increased during the first 6 days and had returned almost to the output from the resting node by day 8 when increases in the production of lymphoblasts and parasitized cells were first detected. The proportions of lymphoblasts and infected lymphocytes increased dramatically during the next 6 days, accompanied by a marked increase in the

Table 2. Lymphoblasts induce mixed lymphocyte responses in autologous peripheral blood leucocytes (PBL) and fractionated efferent lymphatic lymphocytes (ELL) during a lethal infection with *T. parva*.

		Stimulation index: autologous (allogeneic) (days after challenge)			
Stimulator cells infected calves	Responder cells from infected calves	7	9		13
Lymphoblasts	Small ELL	6·4‡	4·5‡	(5·6)‡	7·3‡
from lymph	PBL	0·9	2·4	(6·1)	6·1‡
(Fraction 1)*	ELL	4·4‡	5·3‡	(12·2)‡	5·9‡
Small ELL	Small ELL	1·0	1·6	(1·2)	1·2
	PBL	0·8	2·6	(3·5)	1·6
(Fraction 2)†	ELL	0·6	1·8	(3·4)	0·9
PBL	Small ELL	1·2	2·8	(8·8)‡	4·6‡
	PBL	1·0	2·4	(10·4)‡	2·9
	ELL	1·4	1·9	(12·1)‡	1·4
	Small ELL	1·4	3·9‡	(4·6)‡	4·9‡
	PBL	1·5	3·7	(4·8)‡	1·8
ELL	ELL	1·4	4·2‡	(6·1)‡	0·7

*Fraction 1 contained 60%–70% lymphoblasts with an MSI on days 7, 9 and 13 of $<0{\cdot}1\%$, $2{\cdot}2\%$ and $10{\cdot}8\%$ respectively. Lymphatic lymphocytes were separated as described in Materials and Methods.

†Fraction 2 contained less than 5% lymphoblasts with an MSI on all days of less than 0·1%.

‡SI Significantly increased above responder cell controls ($P<0{\cdot}05$).

Results are expressed as the mean stimulation index of autologous MLRs, with allogeneic MLRs in parentheses. The assay was conducted with lymphocytes separated from five calves. Counts per minute (c.p.m.) in unstimulated cultures of PBL and ELL ranged between 300 and 1200.

Table 3. Cytotoxicity for autologous lymphatic lymphoblasts of peripheral blood leucocytes (PBL) and efferent lymphatic lymphocytes (ELL) during the course of a lethal infection with *T. parva*.

	Percentage specific lysis from autologous lymphoblasts* (Days after challenge)			
Effector cells	13	14	15	16
MLR - ELL†	0	0	6·2	5·8
MLR - PBL†	0	0	7·5	6·2
PBL†	1·1	0·6	2·8	4·7
ELL‡	1·4	2·0	4·6	5·2
Fraction 2‡	0	1·8	5·4	6·1

*Target cells were efferent lymphatic lymphoblasts fractionated from central lymph on the day of the assay as described in Materials and Methods. The macroschizont index (MSI) of the target cells on days 13, 14 15 and 16 was 10·8%, 21·5%, 52·6% and 56·5% respectively.

†Effector cells (PBL or ELL) were harvested from MLRs initiated on days 8, 9, 10 and 11 after challenge. The MLR on each day was established with fractionated lymphoblasts (stimulator cells) harvested from central lymph on the day of the assay. The MSI of the stimulator cells on days 8, 9 10 and 11 was 0·6%, 2·2%, 5·9% and 7·2% respectively.

‡PBL, ELL and fraction 2 of the isolated lymphocytes from central lymph were each prepared from infected cattle on each of days 13, 14 15 and 16 after challenge for assay on the respective day.

Results are expressed as the mean percentage lysis of autologous lymphoblastoid target cells from five calves.

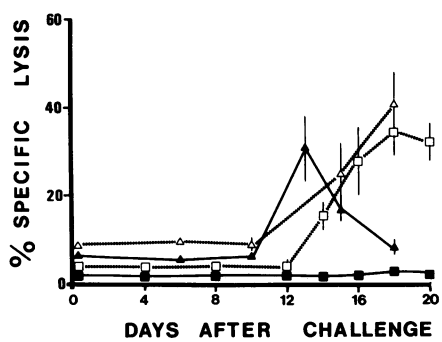

Figure 2. Cytotoxicity of PBL and ELL from infected calves for allogeneic (fifteen calves) and autologous (two calves) lymphoblastoid cell lines infected with *T. parva* and xenogeneic tumour cell line (YAC-1, four calves) during a lethal infection with *T. parva* (all calves died 18 to 22 days after infection). ■ Lysis of autologous target cells (4-hr assay) □ Lysis of allogeneic target cells (4-hr assay) ▵Lysis of allogeneic target cells (12-hr assay) ▲ Lysis of xenogeneic (YAC-1) target (12-hr assay). The assay was established at effector: target (E:T) ratios of 80:1, 40:1, 20:1 and 10:1 (4 hr) and 100;1 and 50:1 (12 hr). Data shown represent the mean percentage lysis of target cells (± 1 SD) at E:T ratios of 40:1 (4 hr) and 50:1 (12 hr).

production of lymph fluid. However, the macroschizont index (MSI) of efferent lympohcytes declined precipitously from 50% on day 15 to less than 0·1% 3 days later. A similar pattern in the percentage and output of blast lymphocytes was also observed. The cellular traffic from the regional lymph node had returned to within normal limits by day 20, and infected lymphocytes could not be detected in lymph after day 17.

Cytotoxicity of peripheral blood leucocytes (PBL) and efferent lymphatic lymphocytes (ELL) from recovering calves. The cytotoxicity for allogeneic cell lines infected with *T. parva* of PBL from three calves and ELL from two calves which recovered naturally from East Coast fever is shown in Fig. 4a. During the third week of the infection, cytotoxic activity for allogeneic infected cells increased in ELL from less than 10% on day 9 to peak at 24·5% on day 18. Spontaneous lysis of allogeneic target cells by ELL preceded and was higher than that of PBL which increased after day 14 to reach a maximum of 10·8% on day 18 after challenge. Thereafter, the cytotoxicity of lymphocytes from both sources declined towards normal levels by day 20.

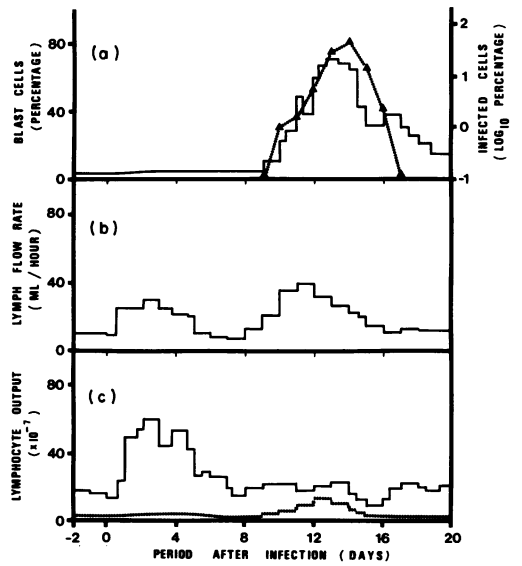

Figure 3. The output of normal and infected lymphocytes from the lymph node draining the site of inoculation of *T. parva* in two calves which recovered from a lethal dose of the parasite.
(a) — Average percentage blast cells in lymph, ▲ average percentage infected cells in lymph (log_{10}); (b) — average lymph flow rate (ml/hr); (c) — average total cell output per hr, ■■■■ average blast cell output per hr.

Both 4- and 12-hr assays yielded similar results. The pattern of spontaneous lysis of xenogeneic YAC-1 tumour cells in the macrocytotoxicity assay was higher in PBL than in ELL, where maximum lysis of 47·3% and 26·5% were recorded respectively on day 18 (Fig. 4a). Unfortunately the unavailability of cell lines precluded an examination of the evolution of autologous cytotoxicity in these calves. Attempts to demonstrate lytic activity in ELL and PBL against autologous infected lymphoblasts isolated from central lymph from day 10 onwards were thwarted by the elimination of the infection after day 16 (Fig. 4a).

Responses mounted by calves during immunization against *T. parva*

Cellular kinetics in central lymph from the regional lymph node. The response to the inoculation of *T. parva* of the draining lymph node of two calves immunized with sporozoites (stabilate) and long-acting tetracycline is shown in Fig. 5. The total cell output and lymph flow rate increased rapidly during the first 5 days after challenge to five times the output of the unstimulated lymph node before declining to normal levels by day 7. Throughout the following week to day 13 the production of lymphocytes and blast cells remained relatively constant. From days 13 to 15 the proportion and output of blast cells in efferent lymph

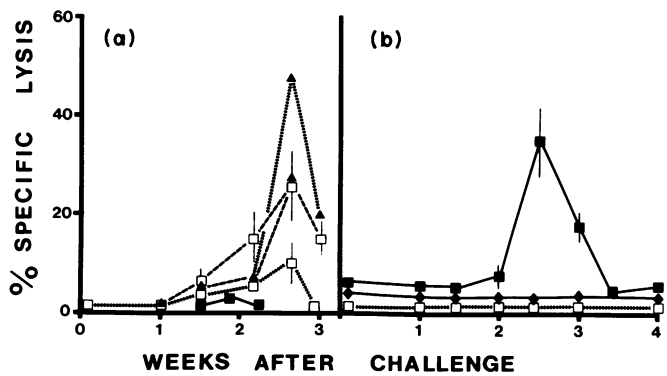

Figure 4. (a) Cytotoxicity for allogeneic and autologous parasitized target cells of PBL from three calves during recovery from a lethal dose of *T. parva*. (b) Cytotoxicity for allogeneic (five calves) and autologous target cells (three calves) of PBL from calves immunized against *T. parva* with stabilate and tetracycline. ■——■ Lysis of autologous target cells by PBL; □· · · · ·□ lysis of allogeneic target cells by PBL; ▲· · · · ·▲ lysis of xenogenic target cells by PBL; □– – –□ lysis of allogeneic target cells by ELL; ▲– – –▲ lysis of xenogeneic target cells by ELL; —— lysis of allogeneic and autologous target cells by PBL stimulated in an MLR with parasitized autologous cells.
Results are expressed as mean percentage lysis of target cells (± 1 SD) from results pooled from both 4-hr and 12-hr assays at effector: target ratios of 40:1 and 50:1 respectively.

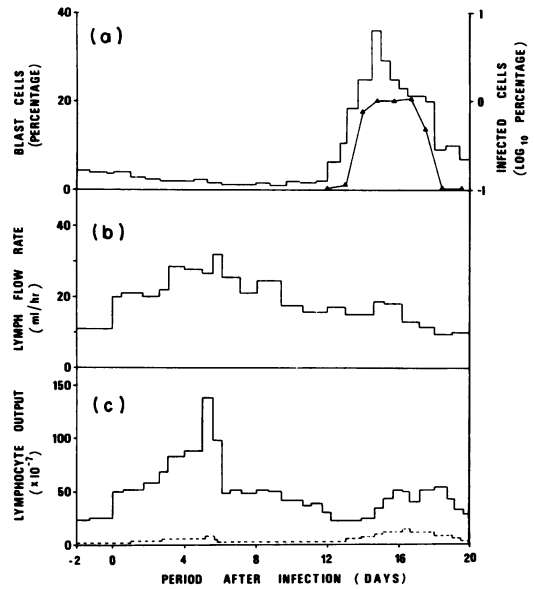

Figure 5. The output of normal and infected lymphocytes from the lymph node draining the site of inoculation of *T. parva* in two calves immunized with stabilate and tetracycline. (a) — Average percentage blast cells in lymph, ▲ average percentage infected cells in lymph (log_{10}); (b) — average lymph flow rate (ml/hr); (c) — average total cell output per hour; ■■■■ average blast cell output per hour.

increased dramatically; the output rose four-fold and the proportion of lymphoblasts increased from less than 5% on day 13 to 35%–40% of total cells 2 days later. Coincidentally macroschizonts of *T. parva* were first detected in lymphoblasts on day 13 (approximately 0·05%) and parasitized cells accounted for 1·0% – 1·2% of total efferent lymphatic lymphocytes collected between 15 and 17 days after challenge. Subsequently the proportion of blast and infected lymphocytes declined; macroschizonts could not be detected after day 19, and blast cells represented less than 10% of the efferent lymphocytes collected after day 18.

Stimulatory capacity for normal autologous PBL in the MLR of lymph node cells (LNC) from immunized calves. The stimulatory capacity for normal autologous and allogeneic PBL of LNC from biopsies of the regional lymph nodes in five calves immunized against *T. parva* is shown in Fig. 6. Throughout the 4 weeks after immunization, LNC-stimulated allogeneic MLR responses that were eight–twelve times higher than those of control PBL. In comparison, the stimulation index (SI) of autologous combinations of LNC and PBL ranged between 1 and 2 for the first 2 weeks after challenge. Subsequently a four–five-fold increase in the autologous MLR response was observed when stimulator LNC were harvested from immunized calves during the third week after challenge. The autologous MLR as maximal when initiated by LNC on days 15 to 17, and declined to normal levels by 23 days. The stimulatory capacity of LNC paralleled the appearance of blast infected leucocytes within the parent lymph node (cf Fig.5).

Cytotoxicity of PBL from calves during immunization against T. parva. Peripheral blood leucocytes obtained from calves during prophylaxis against *T. parva* were tested against autologous (three calves) and allogeneic cell lines (seven calves) infected with *T. parva* in 4-hr and 12-hr assays as shown in Fig. 4b. In contrast to the non-specific cytotoxic activity against allogeneic and xenogeneic target cells of PBL from calves suffering a lethal infection (Fig. 4a), PBL from immunized calves reacted poorly against these target cells (less than 5% lysis) but killed specifically autologous cell lines infected with *T. parva* in the third week after challenge. The specific direct cytotoxicity increased after day 14 from 7·2% to peak 3 days later at

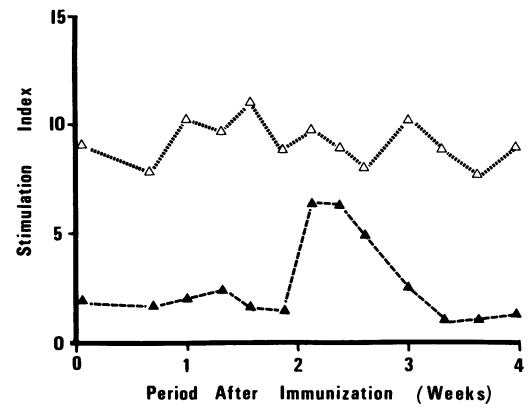

Figure 6. The stimulatory capacity for normal autologous and allogeneic PBL in MLR of cells collected at intervals from the lymph node draining the site of inoculation of *T. parva* in five calves immunized with stabilate and tetracycline. ▲ SI of MLR with autologous PBL (× LNC); △ SI of MLR with allogeneic PBL (× LNC).

Results are expressed as the mean stimulation index (SI) from 4-day cultures and were calculated as described in Materials and Methods. Background counts were 500 to 900 c.p.m.

around 35%. During the ensuing week to day 23, the lytic activity of PBL declined to normal levels.

In comparison, MLRs initiated between responder PBL obtained at intervals during immunization and autologous infected lymphoblasts from cell lines as stimulator cells did to generate detectable cytotoxicity for autologous or allogeneic target cells during the 4 weeks after challenge (Fig. 4b). However, specific lysis of allogeneic target cells by similar PBL established in allogeneic MLRs ranged between 32% and 69% (data not shown).

DISCUSSION

A lethal infection of susceptible cattle with the protozoan parasite *T. parva* was accompanied by the development in host peripheral blood leucocytes (PBL) and lymphatic lymphocytes (ELL) of non-specific cytotoxic activity against allogeneic infected cells and a susceptible xenogeneic tumour cell line (YAC-1). This lytic activity appeared during the third week of infection and was maximal in moribund calves. In two calves which recovered from a lethal dose of *T. parva*, non-specific cytotoxicity disappeared from PBL 2–3 days after the elimination of parasitized lymphocytes.

The lysis exhibited by PBL from calves immunized against *T. parva* was specific for autologous infected cell lines and appeared 14–21 days after infection and treatment. Both this cytotoxicity and the stimulatory capacity in the MLR of lymph node cells from immunized calves occurred when macroschizont forms of *T. parva* appeared in a small proportion of host lymphoblasts.

Following the inoculation of a lethal dose of *T. parva*, macroschizont forms of the parasite are initially detected 5–8 days later coincident with a dramatic cellular blastogenesis in the regional lymph node and its efferent lymph (Morrison *et al.*, 1981; Emery 1981, submitted for publication). Cells harvested from lymph nodes during this period initiate substantial proliferative responses of mixed-lymphocyte type in normal autologous PBL (Emery & Morrison, 1980). In the present study, fractionation of efferent lymphatic lymphocytes (ELL) confirmed that a large amount of the stimulatory capacity of LNC resided in the emergent lymphoblasts. The smaller magnitude of the autologous MLR induced by lymphatic lymphoblasts as compared with LNC (SIs of 10 and 15, respectively) may be associated with both the lesser capacity of ELL than PBL to stimulate a MLR (Emery & McCullagh, 1980) and to the presence of prospective responder cells in addition to infected cells in the lymphoblast fraction employed for stimulation.

Peripheral blood leucocytes and ELL from calves with untreated primary infections with *T. parva* exhibited non-specific cytotoxicity which was maximal around the eighteenth day after inoculation regardless of the final outcome of the disease. Although this lytic activity later disappeared from recovering calves, the magnitude of the cytotoxicity in individual animals was not predictive of death or recovery. Whereas the non-specific cytotoxicity exhibited by PBL in lethal primary infections was high against allogeneic infected cells and low against xenogeneic target cells, the reverse occurred in calves which recovered. Such results might reflect the activity of sub-populations of natural-killer (NK) cells with different specificities in cattle, akin to those described in man (Lotzova, 1980). It is possible that non-specific cytotoxicity might contribute to the lymphocytolysis and panleucopaenia which is prominent during the third week of a lethal infection, but further studies are required to identify both the effector and potential target cells, and to evaluate the contribution of this activity to natural recovery from infections with *T. parva*. Although the cytotoxicity was mediated in assays which detect NK cells in cattle (Rouse & Babiuk, 1977) and other species (Keissling, Klein & Wigzell, 1975) and the genetic spectrum of lytic activity would implicate this cell type, cytotoxicity of PBL from two calves did not extend to autologous target cells. This could result from the retention in lymph nodes of specifically cytotoxic lymphocytes or their precursors throughout the course of a lethal infection. However, it is possible that the presence of unlabelled parasitized lymphoblasts within the effector populations tested during the third week of the infection competitively inhibited the detection of direct autologous cytotoxicity in the respective assays. Attempts to demonstrate cytotoxicity of ELL and PBL for autologous lymphoblasts indicated that before day 14, cytotoxicity had not developed in central lymph or peripheral blood respectively. In contrast, the failure to generate cytotoxicity in autologous MLRs initiated on days 8,9,10 and 11 after infection is probably associated with the requirement for repeated autologous stimulation in MLR of cells from unprimed animals in order to amplify subsequent cytotoxicity (Dausset & Fradelizi, 1977).

It is likely that at least 2 weeks are required after challenge for the maturation in the host of protective

cell-mediated immunity, and that the inductive cells may only present in immunogenic numbers during the second half of this interval. The earliest stage of *T. parva* which induces protective immunity in cattle remains unresolved. Provided that sufficient numbers of uninfected effector cells remain after 14 days to destroy parasitized lymphocytes, then the infection may be eliminated in a fashion exhibited by those calves which recovered from a lethal dose of *T. parva* where the MSI dropped precipitously from 52% on days 15 and 16 to undetectable levels 2 days later. During a lethal infection the parasitosis in lymphoid organs on day 14 is usually in excess of 50% (Morrison *et al.*, 1981), the protective responses would appear clearly outpaced by the replication of the parasite. Moreover, manipulation of the infective dose of sporozoites by dilution or irradiation of stabilate limits the ensuing parasitosis and allows the development of immunity (Cunningham, 1977).

Specifically cytotoxic PBL were obtained from calves during immunization against *T. parva* subsequent to day 14 after infection. Longitudinal studies of several human patients infected with Epstein-Barr virus (EBV) have yielded similar kinetics for the appearance of cytotoxicity in PBL (Svedmyr, Jondal, Henle, Weiland, Rombo, & Klein, 1978). Whereas detectable MLR responses were observed when large numbers of blast and infected cells were present in the regional lymph node and its efferent lymph, the appearance of cytotoxicity with the detection of macroschizonts was probably coincidental. Since the MSI was less than 1·0%, infected cells were probably present in the lymph node before day 14 at levels which were undetectable by immunofluorescence or by stimulation in the autologous MLR. In this respect, it is not known whether the effect of tetracycline on *T. parva* reduced quantifiably the infective dose, the number of parasites which actually matured, or the replication rate of the parasite. In comparison with the other experimental groups, it was evident that the degree of parasitosis declined progressively as lethal infections (maximum MSI 64%–80%) were controlled spontaneously (MSI 52%) or by chemotherapy (MSI 1·0%–1·2%). Whereas extensive parasitoses were associated with the detection of non-specific cytotoxicity, genetically-restricted specific lysis was observed where the severity of the infection was curtailed. The development of specific cytotoxicity would appear to accompany the attainment of immunity, although a causal relationship was not established. However, it is conceivable that if after immunization, non-specific immunity precedes the development of specific cytotoxicity in PBL, the former interactions are confined within the regional lymph node and the activity of PBL reflects the nett results of the initial encounters between host and parasites. These findings are partially analogous with the results of analyses of cytotoxic PBL taken from human patients recovering from infectious mononucleosis (EBV) where both specific and non-specific lytic activity have been demonstrated (Lipinski, Fridman, Tursz, Vincent, Pious & Fellows, 1979; Sugamura & Hinuma, 1980). Unfortunately, autologous cell lines were not available to examine the evolution of specific cytotoxicity in the two calves which eliminated a severe infection. In this respect, calves recovering from Theileriosis exhibit a spectrum of lytic activity similar to that of cattle infected with *Vaccinia* or infectious bovine rhinotracheitis (IBR) viruses (Rouse & Babiuk, 1977). In the latter study, cytotoxicity of PBL remained heterogeneous following a second infection, whereas the lysis of infected target cells by cattle immune to *T. parva* was restricted to the autologous genotype (Eugui & Emery, 1981).

In definitive studies to date, the attainment of immunity to *T. parva* has required the establishment of the parasite in host lymphocytes, as immunity did not develop from inoculation of non-viable sporozoites and macroschizonts (Cunningham, 1977). Transfer of the parasite to lymphocytes of the host sex karyotype has been documented after the inoculation of large numbers of infected allogeneic cell lines (Brown, Crawford, Kanhai, Njuguna & Stagg, 1978). The ability of *T. parva* to reinfect host from donor leucocytes has been suggested as related to the infectivity of the parasite (Cunningham, 1977), but the genetic disparities between the respective leucocytes may also be restrictive. The restriction for the autologous genotype of the cytotoxic response of PBL from immune calves (Eugui & Emery, 1981; this study) is analogous to the specificity of effector cells arising from experimental viral infections in animals and man (reviewed by Zinkernagel, 1979) and suggests a role *in vivo* for cytotoxicity in resistance against reinfection. Therefore, the establishment of *T. parva* in host cells is probably a prerequisite for the induction of specific immune responses which recognise polymorphic host determinants in association with parasite-induced antigen(s) on the surface of infected cells. Moreover, protection against a lethal challenge with *T. parva* can be transferred adoptively between chimeric bovine cotwins with thoracic duct lymphocytes from the immunized partner (Emery, 1980a). At present, there

is no direct evidence that either delayed-type hypersensitivity (DTH) or antibodies play a role in immunity, although DTH reactions have been suggested as operative in recovered cattle (Muhammed, Wagner & Lauerman, 1974). However, more critical evaluation of the respective contributions of humoral antibodies and DTH await the isolation and characterization of the immunogenic complex on the surface of parasitized lymphocytes. Should cell-mediated lympholysis be completely responsible for the immune status of recovered cattle, then the genetic restriction of such responses has obvious implications for immunoprophylaxis of cattle against *T. parva*.

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