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Cell-mediated cytotoxicity in *Theileria annulata* infection of cattle with evidence for BoLA restriction

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SUMMARY

Recovery of calves from tropical theileriosis was accompanied by the disappearance of macroschizonts from lymph nodes and the appearance of cytotoxic cells in the blood and lymph nodes. Acute, fatal disease was associated with incremental parasitosis and parasitaemia and, in general, an absence of detectable cytotoxic cells in the blood or lymph nodes. After recovery from infection, calves were resistant to challenge. Challenge with sporozoites was followed sometimes by an immediate reappearance or by a later peak, or sometimes by twin peaks of cytotoxic cells but macroschizonts were not detected. Histocompatibility (BoLA) typing indicated that calves produced two sequential populations of cytotoxic cells during recovery from primary infection with *Theileria annulata*. The expression of lysis by the first appeared to be BoLA restricted. In contrast, both the peaks of lysis manifest after challenge appeared to be BoLA restricted. Results suggest that BoLA restricted cells are established in the immunological memory and are probably analogous to cytotoxic T cells, while non-BoLA restricted cytotoxic cells are natural killer like cells. The results suggest a role for cytotoxic cells in recovery from primary infection, in the inhibition of proliferation of macroschizonts which evade mechanisms of acquired resistance and in the lysis of macroschizont infected cells deriving from challenge sporozoites which have evaded serum-mediated inhibition.

Keywords cell-mediated cytotoxicity *Theileria annulata* BoLA

INTRODUCTION

Protective immunity to infection with parasites of the genus Theileria in cattle appears to depend on a number of immune responses directed against both extracellular and intracellular stages of the parasite. Thus, serum from immune cattle will inhibit *Theileria annulata* sporozoite infection and transformation of lymphocytes into macroschizont infected, transformed lymphoblastoid cell *in vitro* (Gray & Brown, 1981) and reduce *Theileria parva* sporozoite infectivity for susceptible cattle (Musoke *et al.*, 1982). In contrast *in vitro* studies on both *T. parva* (Pearson *et al.*, 1979; Eugui & Emery, 1981; Emery *et al.*, 1981; Preston *et al.*, 1982) and *T. annulata* (Pearson, 1981) have shown that cell-mediated immune mechanisms may eliminate and/or inhibit the growth of macroschizont infected lymphoblastoid cells.

Studies on *T. parva* reported two types of cytotoxic cell, but have differed as to their record of the frequency of the occurrence of these cell types. Pearson *et al.* (1982) recently confirmed an earlier report (Pearson *et al.*, 1979) that both specific and non-specific cytotoxic cells occurred in cattle

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immune to *T. parva*. Specific killers lysed autologous *T. parva* infected lymphoblastoid cell lines only, whilst non-specific killers lysed xenogeneic as well as allogeneic targets. In contrast, other workers (Emery *et al.*, 1981; Eugui & Emery, 1981) reported the generation of specific cytotoxic cells that would kill self (i.e. autologous *T. parva* infected cell lines) but not non-self (allogeneic cell lines). They presumed that this killing was genetically (MHC) restricted and reported that it occurred only in cattle undergoing immunization against *T. parva*. Non-specific killers, which lysed allogeneic cell lines, occurred only in dying calves and animals revovering spontaneously from severe *T. parva* infection. The latter workers attributed resistance to challenge infections to specific cytotoxic cells. Interpretation of these conflicting results is difficult not only because the two groups used different experimental procedures but also because neither used cattle of defined MHC type. These studies also to some extent ignored the fact that the initial tick derived challenge is with sporozoites. The question of the identity and role of cytotoxic cells in Theileria infections therefore remains controversial.

This paper reports the first study on the nature of cytotoxicity in *T. annulata* infections, and describes the first use of BoLA defined cattle to address the question of whether cytotoxicity in bovine theileriosis is MHC restricted. All data published to date indicates that BoLA (bovine lymphocyte antigen) (Spooner *et al.*, 1979) is the homologue of HLA and H-2 and is the bovine MHC.

This study was undertaken initially to see if the inhibition of growth of *T. annulata* infected, lymphoblastoid cell lines by peripheral blood leucocytes (PBL) of immune cattle was due to a cytostatic or to a cytotoxic effect of the immune cells (Preston, unpublished data). In the first two experiments, PBL from cattle (*Bos taurus*) undergoing immunization against *T. annulata* were cytotoxic, lysing both the autologous and some allogeneic *T. annulata* infected, lymphoblastoid cell lines. This suggested that recognition of *T. annulata* infected target cell lines by cytotoxic PBL might be controlled by BoLA. The third experiment was designed therefore to see if a BoLA restriction occurs in cattle for PBL lysis of *T. annulata* infected, lymphoblastoid cells analogous to the HLA restriction demonstrated in man and the H-2 restriction demonstrated in mice for T cell lysis of virus infected cells (Moss *et al.*, 1981; Zinkernagel & Doherty, 1979).

MATERIALS AND METHODS

Theileria annulata. The Ankara strain, from Turkey (Schein, 1975) and the Hissar strain from India (Gill, Bhattacharyulu & Kaur, 1976) were maintained by passage between the Ludhiana strain of the vector ticks *Hyalomma anatolicum anatolicum* (Gill *et al.*, 1976) and calves infected with tropical theileriosis. Sporozoite suspensions prepared from infected adult ticks were used, either freshly prepared or as cryopreserved stabilates, to infect cells *in vitro* and cattle (Brown, 1979).

Theileriainfected, transformed lymphoblastoid (cell lines). Cell lines were established by infections *in vitro* of bovine PBL from uninfected calves with sporozoites of *T. annulata* harvested from infected ticks (Brown, 1979) and maintained by thrice weekly passage in 10% heat-inactivated (56°C, 45 min) normal calf serum (NCS) in RPMI 1640 with 25 mM HEPES buffer. RPMI 1640 was supplemented routinely with 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 100 μg/ml kanamycin (GIBCO, Europe).

Calves. Ayrshires or Ayrshire × Shorthorn calves (3–6 months of age, 100–150 Kg) were infected by inoculation with stabilates of *T. annulata* sporozoites (2 tick equivalents/calf) in front of the shoulder and maintained on commercial calf pellets and hay at a constant temperature of 20°C. Infection normally resulted in death after 18–24 days. Calves to be immunized were treated 2 days after the onset of clinical reaction (day 10–14) with either halofuginone lactate (Hoechst) at 1·2 mg/kg by mouth, or parvoquone (993C, Wellcome) at 20 mg/Kg intramuscularly. Surviving cattle were challenged with a lethal dose of *T. annulata* sporozoite stabilate.

The clinical condition of the calves was assessed by rectal temperature (pyrexia defined as temperature > 39·5°C), the occurrence of macroschizonts (parasitosis) and lymphoid hyperplasia in the prescapular lymph node draining the inoculation site, and the percentage of erythrocytes infected with piroplasms (parasitaemia) in Giemsa stained smears. The response of calves to

infection with similar doses of *T. annulata* sporozoites varied so much between individuals that their responses could not be pooled. The calves' numbers have been retained in the text to emphasize the individuality of each animal and as a guide to their treatments, cytotoxic responses and patterns of clinical disease (Figs 1–4).

Bovine lymphocyte antigen (BoLA) typing. A microlymphocytotoxicity test was used for BoLA typing (Spooner *et al.*, 1979). Cells were typed for the 16 internationally agreed specificities (designated w), which are alleles at the BoLA-A locus, using internationally recognized antisera (Spooner *et al.*, 1979; Oliver *et al.*, 1981, Anon, 1982). The cells were typed also for other specificities (designated ED) defined by operationally monospecific sera; some but not all of these detect additional putative alleles at the BoLA-A locus. Where tested, the BoLA antigens of uninfected PBL and autologous cell lines were identical, as shown by Spooner & Brown (1981). Calves sharing one or more BoLA antigens were designated matched and calves with no (apparent) common BoLA antigens as mismatched.

Chromium release assay. A ^{51}Cr release microcytotoxicity assay (CRA) was used to detect specific lysis of ^{51}Cr-labelled cell lines incubated with PBL. Significant levels of specific lysis ($P < 0{\cdot}05$) were taken to indicate killing of the target cells by cytotoxic cells.

PBL were harvested from blood withdrawn from the jugular vein into preservative free lithium heparin, 10 u/ml (Sigma) and centrifuged at 2,000*g* for 45 min. Buffy coats were removed, resuspended in phosphate-buffered saline (PBS) (Dulbecco's A medium, Oxoid), layered onto a Ficoll-sodium metrizoate gradient, sp. gr. 1·077 (Lymphoprep, Nyegaard) and centrifuged at 800*g* for 45 min. The cell layer at the interface was removed, cells washed three times in PBS and resuspended at 1×10^7/ml in 10% heat-inactivated fetal calf serum (FCS) in RPMI 1640 (supplemented as described above plus 5×10^{-5}M mercaptoethanol). Target cell lines in 10% NCS in RPM1 1640 (5×10^6/ml) were incubated with 100 μCi sodium chromate (^{51}Cr) solution B.P./ml (specific activity 250–500 μCi/μg chromium, Amersham International) for 1 h at 37°C with intermittent shaking. Cells were washed twice in 10% NCS in RPMI 1640, incubated at room temperature for 30 min to allow release of isotope from dead cells, washed again and resuspended at 2×10^6/ml in 10% FCS in RPMI 1640. Cell viability, assayed by trypan blue exclusion, was greater than 95%. Microcytotoxicity assays were set up by adding 200 μl PBL suspensions (1×10^7/ml) to 20 μl ^{51}Cr-labelled target cell suspensions (2×10^6/ml) in V shaped microtest plate wells (Sterilin), i.e. an effector : target cell ratio of 50 : 1. This ratio had been shown previously to give optimal responses when compared with ratios of 100 : 1 and 25 : 1. Three wells, at least, were set up with each combination of effector and target cell. Plates were centrifuged at 100*g* for 30 s at 15°C, prior to incubation at 37°C in 5% CO_2 in air for either 4 h (4 h-CRA) or 18 h (18-CRA). After centrifugation at 250*g* for 10 min at 15°C, 100 μl supernatant was removed from each well for assay with a γ-counter (Nuclear Enterprises). The specific lysis was calculated from the mean values according to the formula:

$$\text{specific lysis} = \frac{\text{test release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

Counts per minute (ct/min) of target cells incubated with PBL gave test release; with medium alone, spontaneous release; with 1% sodium dodecyl sulphate, maximum release. Significance of the difference between the cpm of test release and the cpm of spontaneous release was analysed by Student's *t*-test; $P < 0{\cdot}05$ was taken to be significant and only where $P < 0{\cdot}05$ have the terms 'lysis, and cytotoxic' been employed.

Assay for specific lysis of T. annulata *cell lines by PBL from calves infected with* T. annulata *(Hissar) and challenged with* T. annulata *(Ankara).* Four susceptible calves were infected with *T. annulata* (Hissar). Two (123, 124) were treated on day 11, two (121, 122) were not; all recovered. PBL were tested in 4 h-CRA at weekly intervals against the Hissar cell line of calf 124. On day 35, calves 122 and 124 plus two more susceptible calves (117, 118) were infected with *T annulata* (Ankara). The former pair resisted this challenge; both susceptible calves died on day 19. As a significant level of specific lysis was recorded only once in a 4 h-CRA during the primary infection (Fig. 1), 18 h-CRA were carried out as well every 3–4 days from day 35. The PBL of each calf were tested then against the Hissar cell lines of calves 122 and 124 and the Ankara cell line of calf 124.

Twenty-eight days after the primary challenge, the two immune calves were challenged again with *T. annulata* (Ankara); both 4 h- and 18 h-CRA were continued for 3 more weeks.

Assay for specific lysis of T. annulata *(Hissar) cell lines by PBL from calves infected and challenged with* T. annulata *(Hissar).* Four calves were infected with *T. annulata (Hissar).* Two (127, 128) resisted this challenge, having recovered spontaneously (without treatment) from a primary infection with this strain given 4 weeks previously. Two (145, 146) were susceptible calves and were treated on days 10 and 12; calf 145 recovered, calf 146 died on day 26. On day 35, calf 128, whose PBL had been demonstrably cytotoxic after challenge, together with calf 145 and two more susceptible calves (147, 148) were infected with *T. annulata* (Hissar). Calf 147 was treated on day 10 and recovered; calf 148 recovered spontaneously. Up to day 35, PBL were tested every 3–4 days in both 4 h- and 18 h-CRA against autologous and allogeneic Hissar cell lines. As significant levels of specific lysis occurred only in 18 h-CRA, 18 h-CRA were used alone after challenge. After challenge, lymph node cells, prepared by teasing and sieving biopsy material withdrawn from the prescapular lymph node draining the inoculation site, were tested in parallel with PBL in an 18 h-CRA, at an effector: target cell ratio of 50:1. BoLA antigens of the cell lines were as follows: *128*:w10, w —, ED 85, ED 101; *145*:w10, w11, ED 85, ED 91; *147*:w6·1, w9; *148*:w6·1, w10, ED 85.

Assay for specific lysis of BoLA-defined T. annulata *(Ankara) cell lines by PBL from calves infected and challenged with* T. annulata *(Ankara).* Four susceptible calves were infected with *T. annulata* (Ankara). Two (154, 155) were treated on day 10; both recovered.Two (153, 156) were not treated; both died on days 14 and 22. On day 28, calves 154 and 155 plus two more susceptible calves (159, 160) were infected with *T. annulata* (Ankara). The susceptible calves were treated; calf 159 recovered; calf 160 died on day 23. Throughout the experiment, PBL from each calf were tested every 3–4 days in an 18 h-CRA against a number of BoLA defined Ankara cell lines. BoLA antigens of calves and cell lines were as follows:

46:w4, w6, ED 85, ED 91, ED 103, ED 639;
124:w7, w10, ED 85, ED 91; *153*:w10, w20, ED 85, ED 108;
154:w2, w —, ED 639; *155*:w2, w2, w —, ED 639;
156:w8, w —, ED 74, ED 87; *159*:w2, w —, ED 104;
160:w8, w11, ED 74, ED 91; *691*:w2, w —.

RESULTS

Specific lysis of T. annulata *cell lines by PBL from calves infected with* T. annulata *(Hissar) and challenged with* T. annulata *(Ankara).*

The overall pattern of specific lysis manifest by PBL from calves 122 and 124 which recovered from infection with the Hissar strain and were subsequently immune to the Ankara strain, indicated that cytotoxic cells circulate in the blood of calves after both primary infection and after challenge with *T. annulata* (Fig. 1) BoLA typing revealed that PBL from these two calves had lysed allogeneic target cells with which they shared four BoLA antigens (calf 122:w7, w10, ED 85, ED 91, ED 105, ED 639; calf 124:w7, w10, ED 85 ED 91). The response of PBL from calf 122 indicated that two populations of cytotoxic cells occurred after challenge (Fig. 1d). Significant levels of specific lysis were demonstrated consistently only when effector and target cells had been cultured for 18 h (Fig. 1b & d). Thus, when PBL harvested during primary infection were tested in 4 h-CRA, only PBL from calf 124 were markedly cytotoxic (Fig. 1c); PBL from calf 123, which also recovered after treatment, were not cytotoxic at all. PBL from calves 121 and 122 (Fig. 1a), which recovered spontaneously, were poorly cytotoxic.

PBL of calves dying from infection (117, 118, 121) lysed neither autologous nor allogeneic cell lines, in either 4h-CRA or 18 h-CRA, except those from calf 118 which lysed the autologous cell line (specific lysis = 20%) in an 18 h-CRA on the day it died.

Specific lysis of T. annulata *(Hissar) cell lines by PBL from calves infected and challenged with* T. annulata *(Hissar)*

The pattern of specific lysis of the lysis of the autologous and allogeneic (matched) cell lines (Fig. 2a

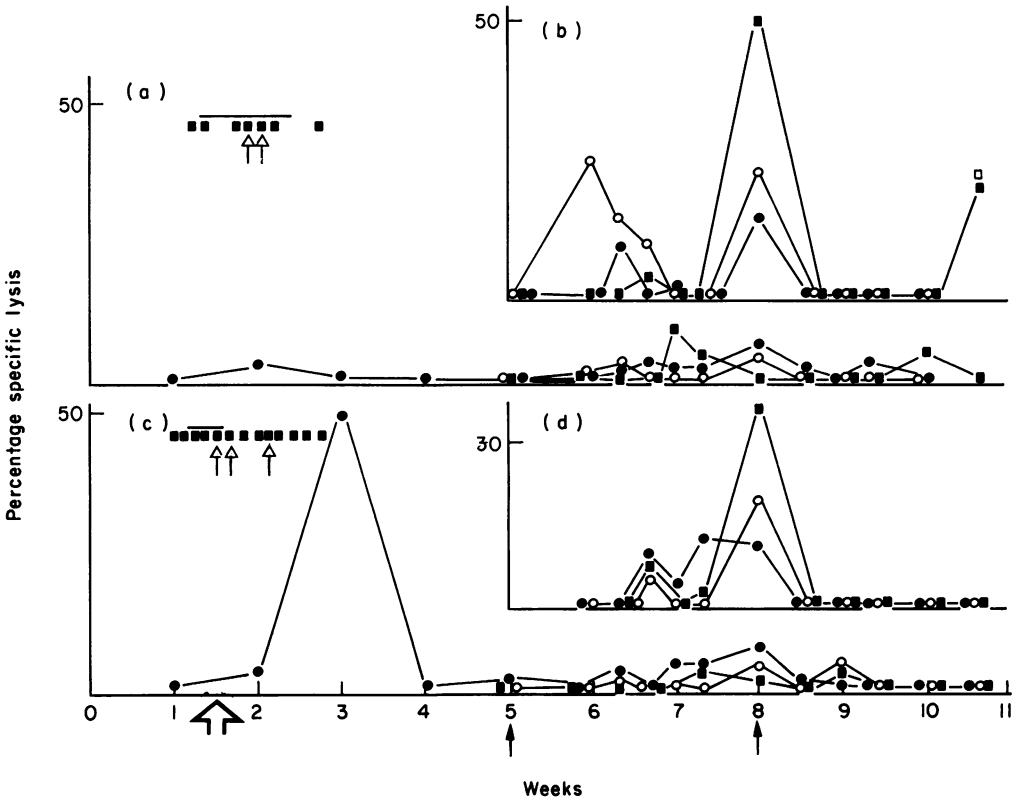


Fig. 1. Percentage specific lysis of autologous and allogeneic Theileria infected lymphoblastoid cell lines by PBL harvested from calf 122 (a, b) and calf 124 (c, d) during primary infection with *T. annulata* (Hissar), and challenge infections with *T. annulata* (Ankara) (a, c) after 4 h-CRA: (b, d) after 18 h-CRA. Cell lines: *T. annulata* (Ankara)—124 (■); *T. annulata* (Hissar)—122 (○); *T. annulata* (Hissar)—124 (●); clinical pattern inset: period of pyrexia ————; days macroschizonts recorded in lymphnodes ■ ■ ■ ■; peak(s) parasitosis ↟. Treatment ↟ (124 only); challenge ↟.

& b) manifest by PBL from calf 145 indicated that two peaks of cytotoxic cells circulate in the blood of calves after primary infection and after challenge with *T. annulata* (Hissar). As no allogeneic (mismatched) cell lines were used in assessing cytotocicity during the primary infection, it was not possible to tell whether the significant levels of lysis demonstrated at this stage were BoLA restricted. After challenge, however, the initial population of cytotoxic cells seemed to be BoLA restricted, lysing only the autologous and BoLA matched cell lines (Fig. 2a & b). The second population lysed a mismatched cell line as well (Fig. 2c).

PBL from calf 146, which died from primary infection on day 25, lysed neither its autologous nor an allogeneic (matched) cell line in either 4 h- or 18 h-CRA.

Only PBL from one of the calves (127, 128), which resisted the primary challenge given when calves 145 and 146 were infected, were demonstrably lytic. Thus PBL harvested 3 weeks after primary challenge from calf 128 lysed the autologous cell line (specific lysis = 40%) but not an allogeneic (mismatched) cell line. PBL harvested 4 days after a second challenge lysed the autologous cell line (specific lysis = 5%) but not the allogeneic (mismatched) cell line. PBL harvested from calf 127 were never demonstrably lytic. The pattern of specific lysis manifest by PBL harvested from the second pair of susceptible calves confirmed that cytotoxic cells occur in calves recovering from infection either spontaneously (calf 148) or after treatment (calf 147). PBL from both calves lysed their autologous (Fig. 3a & e) and allogeneic (matched) cell lines (Fig. 3b & f). Only PBL from

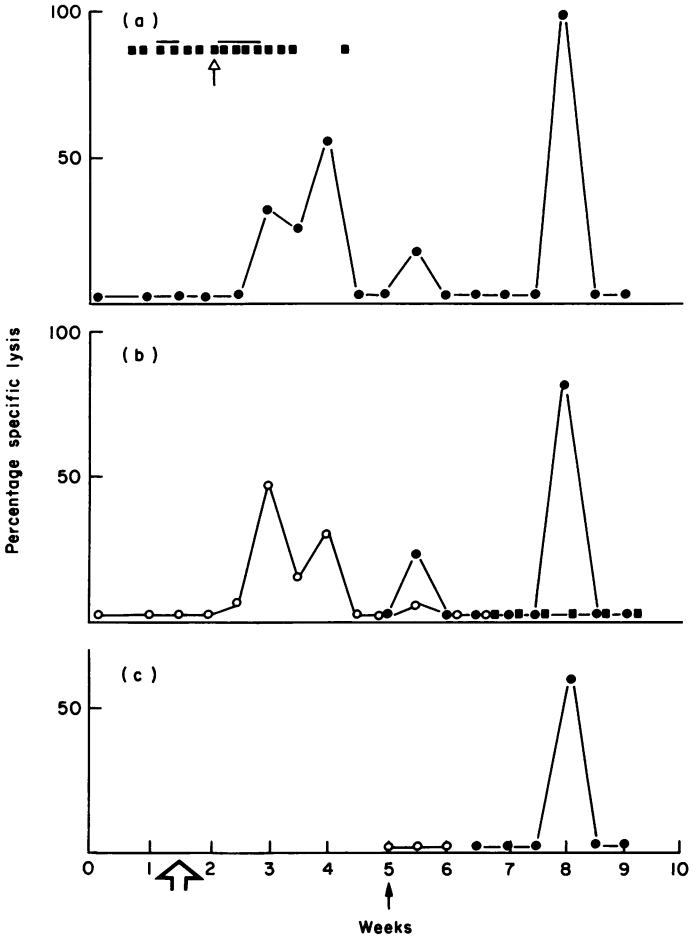


Fig. 2. Percentage specific lysis of autologous and allogeneic *T. annulata* (Hissar) infected, lymphoblastoid cell lines by PBL harvested from calf 145 during primary and challenge infections with *T. annulata* (Hissar) and tested in an 18 h-CRA. (a) Autologous cell line. (b) Matched cell lines of calves 128 (●); 146 (○); 148 (■). (c) Mismatched cell lines of calves 128 (●); 146 (○); 148 (■). (c) Mismatched cell lines of calves 127 (○); 147 (●). Clinical pattern inset: see Fig. 1.

calf 147 were tested against allogeneic (mismatched) cell lines. Only one of the two cell lines was lysed; this was effected by PBL taken during the fourth week of infection (Fig. 3c). Lymph node cells were also demonstrably cytotoxic for both autologous and allogeneic (matched) cell lines (Fig. 3d & g). Two peaks of cytotoxic cells appeared to occur in both the circulation and lymph nodes during primary infections, as seen above (Fig. 2). Thus, the level of specific lysis fell markedly after the initial peak in the second or third week and was rising again when the last assays were carried out.

Specific lysis of T. annulata *(Ankara) cell lines by PBL from calves infected and challenged with* T. annulata *(Ankara)*

PBL from calves 154 and 155, which recovered after treatment from primary infection of the Ankara strain, also manifest two peaks of cytotoxicity during the course of primary infection. Cytotoxic PBL harvested during the first three weeks of infection gave a first peak of specific lysis. These cells lysed both their autologous (Fig. 4a & d) and matched allogeneic cell lines (Fig. 4a, b & e). In contrast, PBL from calf 154 lysed only one and PBL of calf 155 only two of three allogeneic

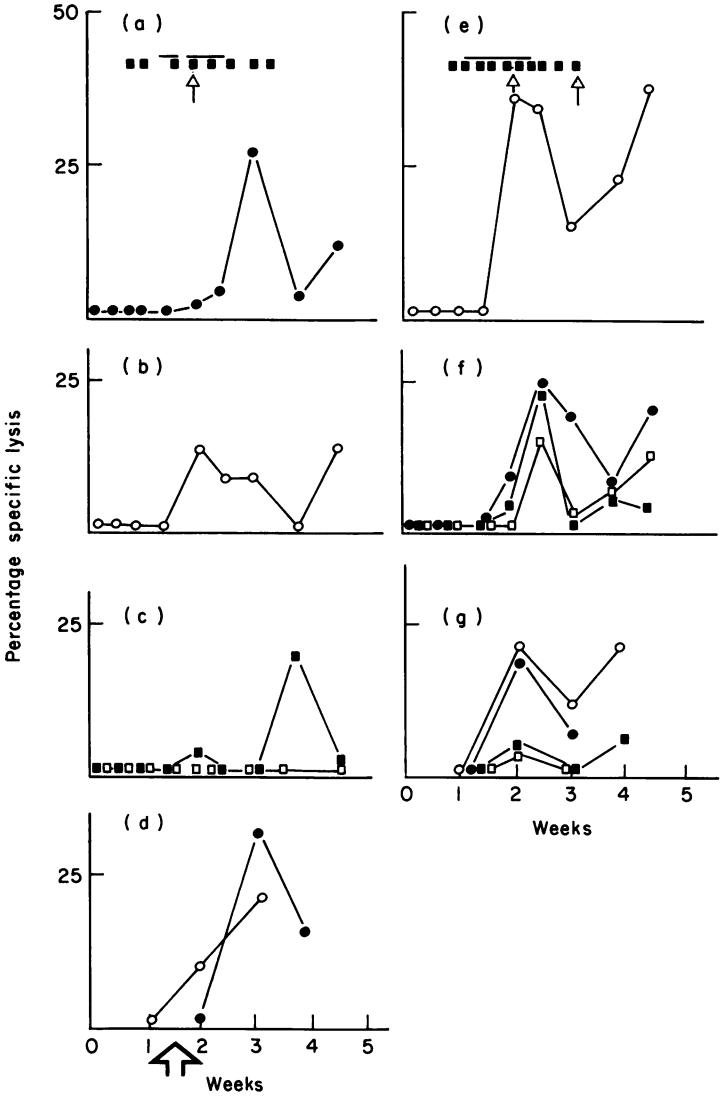
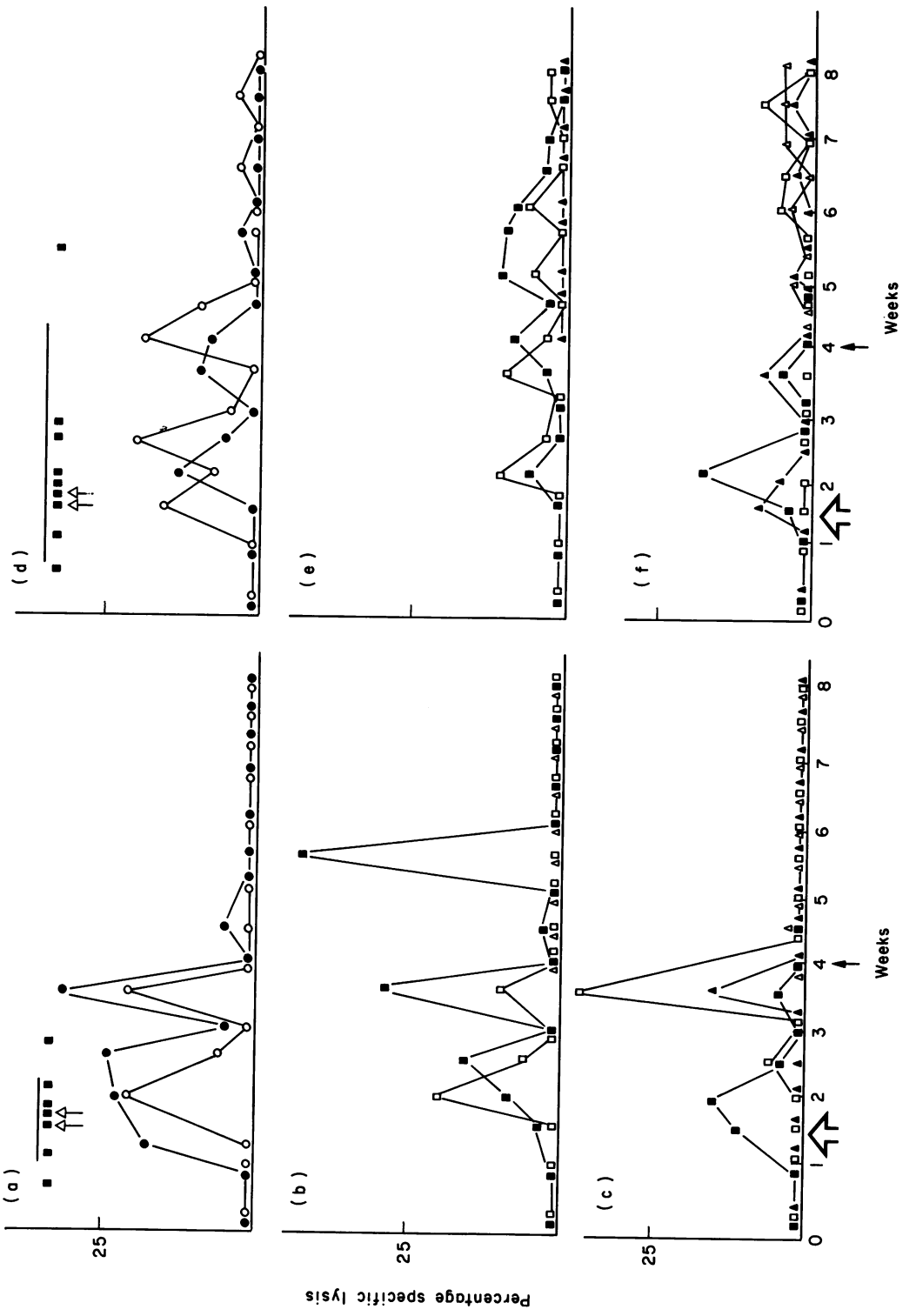


Fig. 3. Percentage specific lysis of autologous and allogeneic *T. annulata* (Hissar) infected, lymphoblastoid cell lines by PBL and lymph node cells harvested from calf 147 and calf 148 during primary infections with *T. annulata* (Hissar) given at the time calf 145 was challenged. PBL tested in an 18 h-CRA. PBL from calf 147 (a, b, c); calf 148 (e, f) tested with autologous cell lines (a, e); matched cell lines of calves (b) 148 (○); (f) 147 (●); 145 (■); 128 (□); mismatched cell lines of calves (c) 128 (■); 145 (□). Lymph node cells (d) from calf 147 tested with the autologous cell line (●) matched cell line of calf 148 (○); (g) from calf 148 tested with the autologous cell line (○); matched cell lines as (f). Clinical pattern inset; (a) calf 147; (e) calf 148. Treatment ⇧. See Fig. 1.

(mismatched) cell lines (Fig. 4c & f). The second peak of specific lysis occurred in the fourth week of primary infection when PBL from calf 154 lysed all three and PBL from calf 155 again lysed two of three allogeneic (mismatched) cell lines, as well as lysing autologous and allogeneic (matched) cell lines.

Although PBL harvested from both these calves during the first week of challenge seemed to be BoLA restricted, i.e. lysing only the autologous and matched allogeneic cell lines (Fig. 4a, b, d & e),


(a)
(b)
(c)
(d)
(e)
(f)
25
Percentage specific lysis
0
1
2
3
4
5
6
7
8
Weeks

PBL harvested later on from these calves showed different patterns of cytotoxicity. PBL from calf 154 lysed only one of three allogeneic (matched) cell lines (Fig. 4b), whilst cells taken from calf 155 manifest low and fluctuating levels of specific lysis against autologous, and both matched and mismatched allogeneic cell lines (Fig. 4d, e & f).

In all, the cell line of calf 155 seemed less susceptible to lysis by PBL from both calves than that of calf 154.

PBL from calf 159, which also recovered from primary infection after treatment, manifest a similar although mardedly weaker pattern of cytotoxicity to that manifest by PBL taken from calves 154 and 155 during primary infection.

In this experiment, the response of calves which died from the infection differed. PBL from the untreated calves 153 and 156, were not demonstrably cytotoxic. PBL from calf 160, which died despite treatment, showed low but significant levels of specific lysis of target cell lines.

Analysis of the relationship between specific lysis and BoLA antigens

The overall patterns of specific lysis suggested strongly that the first peak of lysis during primary infection and the two peaks after challenge were BoLA restricted. The results were analysed therefore (Fig. 5) as in work on Epstein–Barr virus (EBV) (Moss *et al.*, 1981) to see if cytotoxic cells had lysed preferentially the cell lines with which they shared BoLA antigens. The maximum levels of specific lysis of allogeneic target cell lines obtained during the first and second peaks of specific lysis during both primary and challenge infections were calculated as the relative percentage lysis (RPL) of that simultaneously mediated by those same effector cells against the relevant autologous cell line. For the purpose of matching the calves (Figs 1–4) and in this analysis (Fig. 5) all specificities, whether w or ED, were treated equally. Some animals had only one BoLA-A locus allele; the unrecognized allele may have been shared in some cases and not in others, here it was assumed that it was not. The findings are discussed below.

Specific lysis and parasitosis

In six of the eight calves whose primary infections resolved, macroschizonts were first detected in prescapular lymph nodes draining the inoculation site on days 4 or 5 of infection, the day after lymphoid hyperplasia and 2–3 days before pyrexia. Parasitosis increased with lymphoid hyperplasia; time to peak parasitosis differed with individual calves. Macroschizonts were numerous and calves still pyrexic when PBL lysis was manifest first and the first peak of lysis was not attained until after pyrexia had declined. Pyrexia declined before macroschizonts disappeared. The second peak occurred while macroschizonts were decreasing or after they had disappeared. The two other calves recovered from primary infection after prolonged pyrexia; PBL from one (159) manifest two peaks, PBL from the other (155) several peaks of lysis (Fig. 4).

The six calves dying from primary infection manifest persistent parasitosis and pyrexia; PBL of two only were poorly lytic (118, 160) just before death.

Of the eight calves which resisted primary challenge, six did not become pyrexic and macroschizonts were not detected in the prescapular lymph nodes. However, two transient peaks of specific lysis were detected: during the first (calves 122, 145, 154) and third (calves 122, 124, 128, 145, 154) weeks after challenge (Figs 1, 2, 4). No macroschizonts, pyrexia nor lysis were detected in one other calf (127). In contrast, macroschizonts were detected in lymph node smears of calf 155, whose PBL manifest a persistent low level of lysis (Fig. 4). Theileria infected lymphoblastoid cell lines could be isolated from this calf and calf 154, on days 11, 26 and 33 after challenge.

No macroschizonts, lymphoid hyperplasia nor pyrexia were detected in the three calves which resisted a second challenge (128, 124, 122); PBL of one only (122) were demonstrably cytotoxic (Fig. 1).

Fig. 4. Percentage specific lysis of autologous and allogeneic *T. annulata* (Ankara) infected, lymphoblastoid cell lines by PBL harvested from calves 154 and 155 during primary and challange infections with *T. annulata* (Ankara) amd tested in an 18 h-CRA. PBL from calf 154 (a, b, c);); from calf 155 (d, e, f) with autologous cell lines (a, d); (●) matched cell lines of calves (a) 155 (○); (d) 154 (○); (b, e) 46 (□); 159 (▲, △); 691 (■); mismatched cell lines of calves (c, f) 124 (□); 153 (■); 156 (▲); 160 (△). Treatment ⇧; challenge ↑.

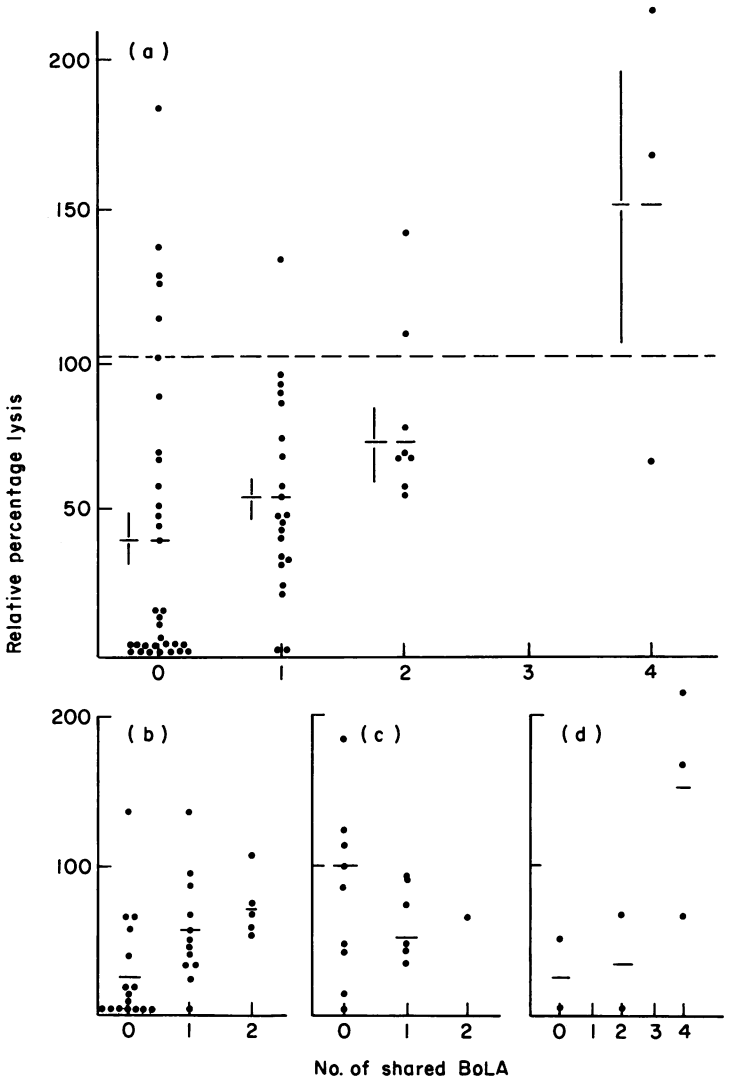


Fig. 5. Relative percentage lysis (RPL) plotted against the number of BoLA shared between effector and target cells. (a) Data for all responses obtained during both first and second peaks of both primary and challenge infection (see Figs 1–4); (b) data from first peak of primary infection; (c) data from second peak of primary infection; (d) data from second peak of secondary infection.

$$\text{RPL} = \frac{\text{specific lysis of allogeneic target cells}}{\text{specific lysis of autologous target cells}} \times 100.$$

$\frac{\bullet}{\bullet}$ individual and mean values; $\frac{|}{|}$ mean values $\pm$ s.e.

$P < 0{\cdot}05$ for differences between means of groups sharing none and four, and, two and four BoLA antigens.

DISCUSSION

This, the first study on cell-mediated cytotoxicity in bovine theileriosis using MHC (BoLA) defined cattle, has provided good evidence that BoLA restricted cytotoxic cells are generated during infections with Theileria. Current work, including this study, does suggest, however, that the ability of cattle to recover from infection and resist challenge infections cannot be attributed to the activity of these cells alone.

Throughout this study on *T. annulata* infections, calves recovering either spontaneously or after chemotherapy from primary infection, possessed cytotoxic PBL and lymph node cells which lysed *T. annulata* infected, transformed lymphoblastoid cells *in vitro*. In contrast, PBL from calves with non-resolving infections were rarely cytotoxic. Both the Turkish (Ankara) and Indian (Hissar) strains gave similar results. It seems likely therefore that cytotoxic cells contribute to recovery from primary infection by eliminating intracellular macroschizonts. Failure to recover from infection may be associated with a multiplication of infected cells insusceptible to the cytotoxic mechanisms of the host, for example as seen with the cell line of calf 155 (Fig. 4a & d).

The brief accelerated response of cytotoxic cells after challenge indicated that populations of cytotoxic cells were established in the immunological memory. This suggests two other roles for these cells: they may (i) inhibit further proliferation of macroschizonts, which evade the mechanisms by which animals recover from infection, and thus prevent relapses of clinical disease; (ii) eliminate lymphocytes infected and transformed by sporozoites which have evaded the humoral response postulated to be the initial defence against tick challenge (Gray & Brown, 1981). Persistence of low numbers of macroschizonts after recovery from infection, i.e. the carrier state, as found in calves 154 and 155, may benefit a host naturally and continually exposed to sporozoites by continually sensitizing the immune system.

Evidence that cattle recovering from infection and challenge with *T. annulata* produced BoLA restricted cytotoxic cells was provided both by overall patterns of specific lysis (Figs 1–4) and by analysing the relationship between the relative percentage lysis (RPL) of allogeneic cell lines and the number of BoLA antigens shared in the different effector:target cell combinations (Fig. 5). Thus, the mean RPL was usually less than 100%, the value for the autologous cell lines. In addition, the mean RPL of the total pool of allogeneic cell lines, which shared one or more BoLA specificities with the effector cells with which they were cultured (i.e. matched), was significantly greater ($P < 0{\cdot}02$) than the RPL of the allogeneic cell lines, which did not share any BoLA antigens with the effector cells with which they were cultured (i.e. mismatched). The level of specific lysis also appeared to be related to the number of BoLA specificities shared by effector and target cells. Thus, the proportion of positive responses increased with the number of shared BoLA antigens and the means of the RPL were directly proportional to the number of shared BoLA antigens.

However, sharing of BoLA antigens did not appear necessary for the expression of all the peaks of lysis. For although the means of the RPL calculated for the first peak of primary infection were directly proportional to the number of shared BoLA antigens (Fig. 5b), the means calculated for the second peak were not (Fig. 5c). Therefore, calves undergoing immunization appeared to produce BoLA restricted cells, manifest as the first peak of lysis, and non-BoLA restricted cells, manifest as the second peak. However, the possibility that theilerial infections may stimulate only BoLA restricted cytotoxic cells is not excluded by these results because the three effector cell populations giving high responses with allogeneic (mismatched) cell lines (Fig. 5b) were from calves (154, 155, 159) for which only one of the two BoLA-A locus alleles has been defined so far. Hence these responses may have reflected sharing of an, as yet, undefined BoLA locus allele.

Both the first and second peaks of lysis manifest by PBL harvested from immune calves after challenge (Figs 2, 4a & 5d) appeared to be BoLA restricted.

The finding that the RPL was proportional to the number of BoLA specificities was reminiscent of the nature of HLA restriction for T cell lysis of EBV infected cells (Moss *et al.*, 1981). Thus the BoLA restricted cytotoxic cells described here, which appeared to be established in the immunological memory, may be T cells as well, recognizing their targets either by a parasite antigen(s) or a parasite-induced neoantigen (Pearson *et al.*, 1979) expressed on target cell membranes in association with BoLA antigens. Lysis of autologous and allogenic cell lines

irrespective of BoLA antigens, by PBL harvested later on in primary infection was reminiscent of lysis by natural killer like cells, which recognize transforming cells *per se* and have been described in other lymphoproliferative diseases, e.g. EBV (Klein & Purtilo, 1981). These results together with those of Pearson *et al.* (1979, 1981) suggest that similar immune mechanisms may be stimulated by both *T. annulata* and *T. parva.*

These results have confirmed the view that, in the absence of BoLA typing or identification of natural killer like cells, cytotoxic cells cannot be classified either as non-specific (i.e. natural killer like) or as genetically (MHC) restricted (i.e. T cell) killers as attempted in some previous studies on genetic restriction in cattle (Rouse & Babiuk, 1977; Emery *et al.*, 1981; Eugui & Emery, 1981). In this study effector:target cell recognition seemed to be restricted both by alleles coded for by the BoLA-A locus and by the broad cross-reacting specificities which have a much wider frequency distribution in particular breeds of cattle than the highly polymorphic BoLA-A locus alleles (Oliver *et al.*, 1981). Examples are ED 85, ED 91 (see Figs 2b & 5e) in Friesans, the breed used in studies on *T. parva.* Thus, lysis of allogeneic (non-self) target cells cannot be ascribed with certainty to non MHC restricted killers, but may reflect sharing of widely distributed broad cross-reacting BoLA specificities, if not BoLA-A locus alleles. In addition, failure to demonstrate killing of an autologous cell line may not reflect an absence of MHC restricted cytotoxicity but rather poor effector:target cell recognition (Rickinson, Wallace & Epstein, 1980) or masking by non-restricted cytotoxicity.

If BoLA restriction is confirmed in the killing of *T. annulata* infected cells, the question then arises as to whether animals with different BoLA types will show differential resistance to tropical theileriosis, as shown for some virus infections in other species (Briles & Olesen, 1971; Zinkernagel & Doherty, 1979) and whether such knowledge could be used in the selection of cattle that could survive in endemic areas of *T. annulata.*

In sum, these studies strongly suggest that PBL from calves immune to *T. annulata* lyse macroschizont infected cells *in vitro* via both BoLA restricted (T?) and non-BoLA restricted (NK?) cytotoxic cells. In addition, macrophages have been shown to inhibit the growth of Theileria infected cell lines *in vitro* (Preston, 1981) and calves undergoing immunization have been shown to possess both the humoral and cellular components of antibody-mediated cellular cytotoxicity (unpublished data). Thus, recovery from primary infection *in vivo* may be mediated by a number of different cellular immune responses directed against Theileria infected cells. It seems likely however that resistance to tick derived challenge in immune cattle is mediated by an initial humoral response directed against sporozoites as well as subsequent cell-mediated mechanisms directed against infected cells.

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