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Initial studies on the properties of a bovine lymphoid cell culture line infected with *Theileria parva*

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SUMMARY

Observations were made on a bovine lymphoblast cell culture line, C2, permanently infected with the protozoan parasite *Theileria parva*. No specific parasite antigen was detected on the C2 cell surface, either by a fluorescent technique or by antibody-dependent cell-mediated cytotoxicity. There was no detectable surface immunoglobulin or secretion of immunoglobulins into the tissue culture medium.

Using immunofluorescence and calf thymus and bone marrow antisera, C2 cells were found to share a membrane antigen with normal calf thymus cells, and also to possess a strong transplantation antigen. Bovine antisera against the latter antigen can initiate killing of C2 cells by normal bovine mononuclear leucocytes in antibody-dependent cell-mediated cytotoxicity. C2 cells can also act as weak stimulators in a mixed leucocyte reaction.

The possible role of such transformed parasite-infected lymphoblasts in the strong functional immunity that follows recovery from *Theileria parva* infection is discussed.

INTRODUCTION

East Coast Fever (ECF) is an acute parasitic disease of cattle caused by the protozoan *Theileria parva*. Within the bovine host, the life cycle of the parasite is characterized by two stages, a schizont stage within cells of lymphoid origin and a piroplasm stage within erythrocytes.

Work by Malmquist, Nyindo & Brown (1970) resulted in the establishment of a continuous cell line of *T. parva*-infected lymphoblasts, derived from the spleen of an ECF-infected cow. This cell line, referred to as C2, has been termed transformed (Malmquist *et al.*, 1970) on the basis of marked motility, increased replication time and the ability to grow in suspension, properties not seen in cultured normal bovine spleen cells.

Brown *et al.* (1971) showed that 80% of cattle receiving optimal doses of C2 cells were immune to a lethal ECF challenge. However, the use of C2 cells as a potential vaccine is complicated by the introduction of large numbers of allogeneic bovine lymphoid cells into the recipient host. This results in a marked anti-lymphocyte response by the host against the C2 cells (Wagner & Duffus, 1974). Such histo-incompatibility could influence the successful establishment of the parasite within the host, a prerequisite for immunity (Duffus & Wagner, 1974; Wagner *et al.*, 1975).

Studies on other lymphoid cell cultures have examined properties such as surface immunoglobulin (Moore & Minowada, 1973), the production of immunoglobulin (Moore & Minowada, 1969), the presence of thymus dependent (T), or thymus independent bone marrow derived (B) functions (Han & Minowada, 1973) and the ability to stimulate a mixed leucocyte reaction (MLR) (Koide & Takasugi, 1976).

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The experiments reported here describe a preliminary study of C2 cells involving some of the above techniques, as well as the use of antisera to examine the nature of antigen expressed on the C2 cell surface and the use of C2 cells as targets in antibody-dependent cell-mediated cytotoxicity (ADCC).

MATERIALS AND METHODS

Cell culture. The *T. parva*-infected C2 cell line of Malmquist *et al.* (1970) was used. Cells were grown in Eagle's MEM with Eagle's salt base (Grand Island Biological Co., New York) supplemented with 0·01 g L-asparagine per litre, penicillin at 100 u/ml, dihydrostreptomycin at 100 μg/ml (MEM) and 20% heat-inactivated foetal calf serum (MEM/FCS). After 3–4 days incubation at 37°C cells were harvested by centrifugation at 250 *g* for 5 min and washed twice in MEM/FCS before use.

Antisera. Rabbit anti-bovine T, anti-bovine B and anti-C2. Bos taurus calves aged from 4 to 10 days old were humanely killed. The thymus was aseptically removed into 0·15 M phosphate buffered normal saline solution, pH 7·2 (PBS) and a cell suspension prepared by gentle mincing with scissors. Bone marrow suspensions were obtained by removing the marrow from the sternum of the calf and washing it repeatedly with PBS. Both cell suspensions were filtered through a 30 μ stainless steel mesh and washed three times with PBS. C2 cells were similarly washed with PBS.

Swiss-albino rabbits, approximately 6 months old, were inoculated with 10% (v/v) suspension of cells in PBS mixed with Freund's complete adjuvant (two parts cells to one part adjuvant); the inoculum (0·2–0·4 ml) was given into intramuscular, subcutaneous and intraperitoneal sites. Two weeks later similar injections were given and the rabbits exanguinated after a further 10 days. The serum was separated and inactivated at 56°C for 30 min.

To absorb the sera, 2 vol. of packed cells (either C2, thymus or bone marrow) were added to 1 vol. of neat serum and incubated at 37°C for 60 min. After centrifugation at 800 *g* for 10 min, the supernatant was mixed with another 2 vol. of cells, incubated for a further 60 min and then left for 18 hr at 4°C. Finally, the serum was removed by centrifugation and stored at −20°C. The three antisera against thymus, bone marrow and C2 cells were absorbed with both the homologous and the two heterologous cell suspensions.

Bovine anti-T. parva and anti-C2. Sera were collected from *T. parva* immune cattle 30 days following a lethal ECF challenge. Such sera had high titres of *T. parva* antibody both in serological assays (Wagner *et al.*, 1975) and in ADCC against chicken erythrocytes coated with *T. parva* antigen. Full details of the latter test are published elsewhere (Duffus *et al.*, 1978), but the above antisera showed a significant destruction of the antigen coated erythrocytes at dilutions up to 10^6.

Bovine anti-C2 sera were obtained as previously described (Wagner & Duffus, 1974). Some *T. parva* and C2 antisera were precipitated with ammonium sulphate and the crude Ig conjugated with fluorescein iso-thiocynate (FITC), according to the techniques of Clark & Shepard (1963).

Rabbit anti-bovine Ig and sheep anti-rabbit Ig. Bovine IgG_2 prepared from normal serum by DEAE fractionation (Duncan *et al.*, 1972) was emulsified with Freund's complete adjuvant in a 2:1 ratio. Rabbits were given an intramuscular injection of 1·0 mg IgG_2 followed by a similar injection 4 weeks later, and exanguinated after a further 10 days. On immunoelectrophoresis (Duffus & Wagner, 1974), the antisera had activity against bovine IgM, IgG_1 and IgG_2. A sheep antisera to rabbit IgG was similarly prepared.

Both antisera were precipitated with ammonium sulphate and the crude Ig conjugated with FITC (Clark & Shepard, 1963).

Fluorescent antibody tests. For the indirect fluorescent antibody (IFA) tests, absorbed and unabsorbed rabbit antisera against bovine thymus, bone marrow and C2 cells were used. A 0·2 ml aliquot of cell suspension, containing 2×10^6 cells/ml in PBS with 1·0 mM sodium azide, was mixed with 0·2 ml of an antiserum dilution and left for 30 min in a melting ice-bath. The cells were then washed three times in PBS containing sodium azide before 0·1 ml of FITC-labelled sheep anti-rabbit Ig (1·0 mg protein/ml) was added. The cells were left for 5 min in melting ice before the excess conjugate was removed with one wash in PBS and sodium azide. The cell preparations were then examined under a fluorescent microscope (Zeiss Model 14). Cells showing membrane fluorescence (Von Fellenberg, Briner & Guggisberg, 1971; Taylor *et al.*, 1971) were scored as positive.

The same IFA technique was used with bovine *T. parva* and C2 antisera, followed by FITC-labelled rabbit anti-bovine Ig, as well as direct fluorescent antibody tests using the same bovine antisera labelled with FITC.

Radial immunodiffusion. Bovine immunoglobulin levels in spent medium were determined by radial immunodiffusion as described by Cowan & Wagner (1970). Medium was collected from replicate cultures on successive days by centrifugation at 800 *g* for 10 min, and membrane filtration (0·5 μ) of the supernatant. Dilutions of medium in agar were prepared in 35 mm diameter plastic cups (Linbro Chemical Co., USA). After the agar had solidified, 3·6 mm wells were cut and 8 μl of the rabbit anti-bovine Ig were added. After 48 hr at room temperature in a humid box, the diameter of the precipitin rings around the wells was measured.

Preparation of bovine mononuclear cells. Venous blood was collected in EDTA (disodium salt) at a concentration of 2 mg/ml and centrifuged at 2300 *g* for 15 min. The crude buffy coat was removed and layered onto 3·0 ml of a solution of 65 parts 8% Ficoll (Pharmacia) and 35 parts of 32·8% sodium metrizoate (Nyegaard, Oslo) and then centrifuged at 1200 *g* for 13 min. The interface was resuspended in MEM containing 1·0 mg/ml EDTA and the platelets removed by washing the cells twice with the latter medium and twice more with MEM, before resuspension in MEM plus 10% autologous serum.

Mixed leucocyte reaction. A unidirectional MLR was done by co-culturing 1×10^6 responder lymphocytes with 1×10^6 or 1×10^5 mitomycin C treated stimulators (25 μg/ml for peripheral leucocytes or 50 μg/ml for C2 cells, at 37°C for 60 min) in 1·0 ml MEM containing 10% autologous serum (autologous for responder lymphocytes). The cultures were incubated at 37°C in sterile 2·5 ml flat-bottomed plastic tubes (Luckham Ltd) previously sulphonated according to the method described by Paul (1973).

After 6 days, 25 μl of MEM containing 2μCi of ^{3}H-thymidine (Radiochemical Centre, Amersham) was added, the tubes vortexed and incubated for a further 150 min at 37°C. The cells from each culture were then harvested onto 2·5 cm GFA discs (Whatman) using a Millipore sampling manifold, washed once in PBS, followed by two washes with 10% trichloroacetic acid and three washes in methanol. The discs were dried in glass vials, 10 ml of scintillation fluid (0·6% 2·5-Diphenyloxazole in toluene) added and the amount of tritium estimated in a liquid scintillation counter (Packard).

A 'blastogenic index' was calculated according to Han & Minowada (1973) as counts per minute (ct/min) in the mixed-cell culture minus ct/min in the mitomycin stimulator control, divided by ct/min in the responder lymphoid cell control.

Antibody-dependent cell-mediated cytotoxicity. This assay is described in detail elsewhere (Duffus *et al.*, 1978). Briefly, bovine mononuclear cells were purified from peripheral blood as described above. For the targets, C2 cells of passage 57 were harvested and washed twice in Eagle's MEM with Hanks's salt base containing penicillin (100 u/ml), dihydrostreptomycin (100 μg/ml), 20 ml HEPES and 10% foetal calf serum (MEM-H/FCS). The cells were resuspended to $1\cdot6\times10^7$ cells/ml. Labelling was carried out in less than 0·5 ml in conical-based plastic centrifuge tubes. Sodium chromate (^{51}Cr, Radiochemical Centre, Amersham) was added at 50–100 μCi/10^6 C2 cells. After incubation for 60 min at 37°C, the cells were washed six times in MEM-H/FCS.

The targets, antisera and effector cells were diluted in MEM-H/FCS at an effector cell to target ratio of 50:1, and each cytotoxicity assay was performed in 0·3 ml vol. in four replicate tubes, according to the technique of Butterworth *et al.* (1974). The tubes were incubated for 6 hr at 37°C before the contents were vortexed and centrifuged at 250 *g* for 5 min. Half of the supernatant (0·15 ml) was then withdrawn into a fresh tube and both the supernatant and pellet (the latter containing half the supernatant and the pellet) were counted for ^{51}Cr.

Maximum release from the target cells was determined by three cycles of freeze-thawing. The percentage isotope release was calculated for each tube from the supernatant (S) and pellet (P) tubes, after correcting for background, by applying the equation:

$$\text{Percentage release} = \frac{S\times2\times100}{S+P}.$$

A percentage cytotoxicity figure was then derived from the following equation:

$$\text{Percentage cytotoxicity} = \frac{\text{percentage released in test} - \text{percentage released in medium}}{\text{maximum percentage release} - \text{percentage released in medium}}\times100.$$

RESULTS

Fluorescent antibody tests

Table 1 shows results of both indirect and direct FA tests using bovine *T. parva* and C2 antisera with calf thymus and C2 cells. The *T. parva* antisera were negative against both cell populations, although

TABLE 1. Staining of C2 or calf thymus cell membranes using bovine *T. parva* or C2 antisera

	Indirect*						Direct†			
Antiserum	6828	A98	102	6835	823	837	6828	A98	923	837
Specificity	*T.p.*‡	*T.p.*	*T.p.*	*T.p.*	C2	C2	*T.p.*	*T.p.*	C2	C2
Fluorescence with thymus cells	−	−	−	−	++	+++	−	−	+++	+++
Fluorescence with C2 cells	−	−	−	−	+++	+++	−	−	+++	+++

* Indirect fluorescent antibody test using bovine antisera at 1:20 and rabbit anti-bovine Ig conjugated with FITC.

† Direct fluorescent antibody test using FITC-conjugated bovine antisera.

‡ *Theileria parva.*

− = No fluorescence.

\+ to +++ = Increasing level of fluorescence.

all had high titres of specific activity against both piroplasm and schizont antigens in serological assays, or against piroplasm antigen in ADCC. Conversely, C2 antisera show strong fluorescence with both the homologous C2 cells as well as calf thymus cells.

Using FITC-labelled rabbit anti-bovine Ig, between 20% and 30% of circulating bovine lymphocytes showed brightly staining rings in the presence of sodium azide and the capping phenomenon (Taylor *et al.*, 1971) in its absence. No fluorescence occurred with C2 cells and approximately 0·2% of thymus cells stained, possibly due to contamination with circulating leucocytes.

Table 2 shows results using both the unabsorbed and absorbed rabbit antisera to bovine thymus, bone marrow and C2 cells. Homologous absorption removed specific staining. After heterologous absorption, the anti-thymus and bone marrow sera showed specificity for homologous cells at dilutions above 1:20, the thymus cell antiserum showed positive staining up to 1:1280, the bone marrow antiserum up to 1:160. Using the specifically absorbed antisera, staining of C2 cells was obtained only with the thymus antisera. Absorption of the thymus antisera with C2 cells removed this activity but not all the activity against thymus cells. The unabsorbed C2 antisera showed positive staining with both C2 and thymus cells and, as with the unabsorbed thymus antisera, a faint staining of bone marrow cells. The specific anti-thymus cell activity was removed after absorption with thymus cells, but the staining of C2 cells with this antiserum remained undiminished. When C2 antiserum was absorbed with C2 cells, activity against the C2 as well as thymus cells was lost.

TABLE 2. Immunofluorescent results using bovine lymphoid cells and rabbit anti-lymphocyte sera*

Rabbit antisera (1:20 dilution)	Thymus	Bone marrow	C2 cells
Anti-thymus	+++	±	++
Anti-thymus absorbed thymus	−	±	−
Anti-thymus absorbed bone marrow	+++	−	++
Anti-thymus absorbed C2	++	±	−
Anti-bone marrow unabsorbed	+	++	−
Anti-bone marrow absorbed thymus	−	++	−
Anti-bone marrow absorbed bone marrow	−	−	−
Anti-bone marrow absorbed C2	n.d.	n.d.	n.d.
Anti-C2 unabsorbed	++	±	+++
Anti-C2 absorbed thymus	−	−	+++
Anti-C2 absorbed bone marrow	++	−	+++
Anti-C2 absorbed C2	−	−	−

* Indirect FA test with FITC-conjugated sheep anti-rabbit Ig.
− = No fluorescence.
± = Faint fluorescence.
+ to +++ = Increasing level of fluorescence.
n.d. = Not done.

Immunoglobulin production

Radial immunodiffusion tests were performed on spent media collected from replicate C2 cultures from 1 to 10 days of culture. Original medium was used as a control. At media dilutions of 1/5 and 1/10 in the agar, the application of 8·0 μl of rabbit anti-bovine Ig resulted in precipitin rings of 4·5 and 5·5 mm in diameter, representing the globulin component of the FCS. Reactions of spent medium at the above as well as other dilutions revealed no increase in the Ig concentration.

Mixed leucocyte reaction

Table 3 shows a unidirectional MLR between bovine responder peripheral blood lymphocytes and mitomycin C treated C2 cells or bovine peripheral blood leucocytes. Both the latter cells induced an MLR

TABLE 3. Unidirectional mixed leucocyte reaction* of bovine responder lymphocytesa gainst mitomycin C-treated leucocytes or C2 cells

	^{3}H-thymidine incorporation (ct/min)			
1×10^6 Responder lymphocytes	Stimulating cells		MLR	Blastogenic index
203	1×10^6 PBL	390	11,054	52·5
	1×10^5 PBL	81	5818	28·3
	1×10^6 C2	1142	5346	20·7
	1×10^5 C2	44	3357	16·8

* Culture maintained at 37°C for 6 days.
† Bovine peripheral blood leucocytes.

with allogeneic lymphocytes, although stimulation with peripheral blood leucocytes produced larger blastogenic indices than C2 cells, especially at a responder to stimulator ratio of 1:1. One feature of C2 cells was the relatively high ct/min in the 1×10^6 C2 cell control; increasing the concentration of mitomycin C above 50 μg/ml did not reduce this count.

Antibody-dependent cell-mediated cytotoxicity

Results of an experiment involving both *T. parva* and C2 antisera, bovine peripheral blood leucocytes as effector cells and C2 cells as targets, are shown in Table 4. The isotope released is expressed as a percentage cytotoxicity; the C2 antisera were cytotoxic up to dilutions of 1:500, whilst even at the highest concentration of *T. parva* antisera, no cytotoxic antibody was detected. This is in agreement with the FA studies where no surface fluorescence of C2 cells occurred using *T. parva* antisera, in contrast to the positive fluorescence seen with antisera from cattle infected with C2 cell culture material.

TABLE 4. Cytotoxicity against the C2 cell line

		Percentage cytotoxicity* at serum dilutions of:			
Serum	Source	1:5	1:50	1:500	1:5000
F446	36 days after inoculation of 10^8 C2 cells	9·4	11·2	2·7	0·9
F203	24 days after inoculation of 10^8 C2 cells	11·3	10·4	0·5	0·1
F468	32 days after inoculation of 10^8 C2 cells	13·1	13·1	3·2	0·4
F193	24 days after inoculation of 10^8 C2 cells	12·5	12·7	10·3	2·9
6658	30 days after *T. parva* challenge	0·1	0·5	0·1	0·7
102	30 days after *T. parva* challenge	0·2	0·9	—	—
A98	30 days after *T. parva* challenge	—	—	—	—
6674	30 days after *T. parva* challenge	0·4	0·6	0·9	—

* Isotope release was measured after 6 hr of incubation with an effector cell to target ratio of 50:1.

DISCUSSION

T. parva is normally transmitted by ticks of the genus *Rhipicephalus*. Sera from animals recovering from a tick-derived infection and later challenged with further tick infective material, were used in attempts to demonstrate parasite antigen on the surface of the *T. parva*-infected cell line, C2; no antigen was demonstrated either by direct or indirect FA techniques. These *T. parva* antisera had very high titres of specific antibody in serological assays, as well as positive results in ADCC against piroplasm antigen coated chicken erythrocytes, at serum dilutions up to 10^6.

The C2 cell itself engendered high titres of specific antibody when inoculated into cattle or rabbits. Such antisera, after absorption with normal bovine lymphoid tissues, retained their activity against surface antigens on the C2 cell. The phenomenon of lymphoblasts in cell culture synthesizing antigens not present on normal lymphocytes has been demonstrated and discussed by Thomas & Edwards (1973) and Kano *et al.* (1972). Viral infection (such as EB virus) may possibly play a role in bringing about the appearance of new cell membrane antigens (Klein *et al.*, 1967), and there is evidence that such cell cultures acquire alloantigens independent from the HLA system (Kano *et al.*, 1972; Koide & Takasugi, 1976). The lack of knowledge on the surface antigens of bovine leucocytes and the shortage of suitable markers makes similar research on bovine cell culture lines very difficult. However, for ECF the establishment of autologous *T. parva*-infected lymphoblast cell cultures from individual bovines by *in vitro* infection should help elucidate the problem. By using the culture cells as targets, the immune response following recovery from infected tick challenge may be studied.

C2 cells were also shown to possess a membrane antigen shared with calf thymocytes. Cultured lymphoid cell lines established for other species, especially the human, both from normal individuals and those with neoplasia, have often been classified either as B cell derived (Moore & Minowada, 1973) or as T cell derived (Han & Minowada, 1973), or even B and T cell lines derived from one individual (Koide & Takasugi, 1976). We have studied only the C2 cell line; other *T. parva* infected lymphoblast cultures exist and there is a strong possibility that such cells may manifest different membrane antigens.

Published work on MLR using lymphoblast cell lines as stimulators has produced conflicting results. Some authors (Han & Minowada, 1973) found that while B cell lines could stimulate, 'leukaemic' T cell lines could not. However, Callewaert *et al.* (1975) have shown that a T cell line could stimulate an MLR, but more than four times as many cells were needed. Our results show that whilst C2 cells can stimulate normal bovine peripheral lymphocytes, the reaction is weaker in comparison with normal peripheral leucocytes used as stimulators.

In ADCC the C2 cells are susceptible to bovine cytotoxic antibody and bovine effector cells, but only with sera from cattle infected with C2 cells and not with sera from cattle infected with tick-derived material. These results reiterate the lack of demonstrable *T. parva* antigen on the C2 cell surface.

There is also a possibility that antigens are absorbed onto the surface of C2 cells during culture. For example, proteins from foetal calf serum have been found on tissue culture cells (Eng & Landon, 1971). However in the present study no specific staining was found using a FITC conjugated rabbit anti-bovine Ig which, in control preparations, stained peripheral Ig-bearing lymphocytes. In addition, radial immunodiffusion did not demonstrate the production of Ig into spent tissue culture medium, but the technique may not be sufficiently sensitive to detect very small differences in Ig concentration.

In *T. parva* infection, the parasite invades lymphoid cell populations and the resultant infected lymphoblasts could stimulate the host to mount an immune response against transplantation antigens present on the surface of the lymphoblasts. On subsequent parasite challenge, i.e. re-invasion of lymphoid cells, an anamestic response against these transplantation antigens, whether mediated by a mechanism such as ADCC or not, could stop further development of the parasite. Such an immune mechanism may in part explain the strong functional immunity which exists in cattle recovering from *T. parva* infection. A final point concerns the practical importance of utilizing *T. parva*-infected cell lines, such as C2, for a potential vaccine, as the resultant anti-lymphocyte response in recipient bovines might endanger the success of subsequent inoculations based on bovine material.

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