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ORIGINAL PAPER

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A dual role for immunosuppressor mechanisms in infection with *Theileria annulata*: well-regulated suppressor macrophages help in recovery from infection; profound immunosuppression promotes non-healing disease

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Abstract There is increasing evidence that immune mechanisms are involved in the pathogenesis of many parasitic infections, including infections with the tick-borne protozoan *Theileria annulata*. The initial stages of tropical theileriosis are characterised by the induction of a non-specific lymphoproliferation by schizont-infected cells which is believed to disrupt antigen recognition and interfere with protective immune responses. This study examined the possibility that cattle do not always succumb to infection because macrophages suppress this non-specific lymphoproliferation. The results provide evidence that lymphoproliferation in cattle may be controlled by two types of suppressor macrophages. The first type occurs in infected cattle and acts via a feedback loop well documented in other parasitic infections. This loop involves macrophages, apparently activated by high levels of gamma interferon produced by proliferating lymphocytes, which suppress lymphocyte proliferation via a prostaglandin-mediated pathway. The properties of a suppressor activity seen in immunised and challenged animals suggested that cattle also possess a type of novel suppressor macrophage recently described in filarial infections. This second type of suppressor macrophage does not seem to act via prostaglandin; its activity appeared to be linked to a suppressor epitope on the sporozoite antigen SPAG-1. Differences in the nature of the schizont-infected cells of the Friesian and Sahiwal calves used in one section of this work, in the in vitro and in vivo lymphoproliferative responses of the two groups of calves and in the behaviour of their suppressor macrophages suggested several reasons why the outcome of *Theileria* infections differed in the two cattle breeds. This study has extended our knowledge of the pluripotential activities of macrophages in *T. annulata* infections to include immunosuppression as well as anti-parasite responses and confirmed the view that the outcome of infection with *T. annulata*, as with many parasitic infections, depends upon the final balance of the protective and pathological properties of the immune system.

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Introduction

While *Theileria annulata* (Dchunkowsky and Luhs 1904) clearly induces very effective protective immune responses in cattle (Preston et al. 1999), tropical theileriosis causes morbidity and loss of productivity in indigenous cattle and severe, and often lethal, disease in imported and high-grade crossbred stock throughout endemic areas (Brown 1997). The response to infection is clearly influenced by the virulence (Sergent et al. 1945) and dose (Preston et al. 1992) of the parasite. High doses of sporozoites may cause acute lethal disease in susceptible hosts; death may follow the proliferation of schizont-infected cells or the haemolytic anaemia associated with intra-erythrocytic piroplasms. Recovery from infection is accompanied by the development of solid, long-lasting immunity to sporozoite challenge. Differences in cattle breed also appear to influence susceptibility to infection, but it is not known why (Preston et al. 1992).

Knowledge of the pathogenic mechanisms that underlie tropical theileriosis is scanty but there is evidence that *T. annulata* schizont-infected cells play a direct role in the failure of protective immune responses by inducing a 'non-specific' proliferation of lymphocytes and disrupting antigen recognition and effector mechanisms during the initial stages of infection (Campbell et al. 1995). The sequence of events may be represented by two in vitro systems. In the first, naive, uninfected lymphocytes proliferate during the initial establishment of *T. annulata* schizont-infected cell lines as trophozoites transform into schizonts, the level of proliferation of the uninfected lymphocytes being proportional to the numbers of infected cells (Preston and Brown 1985). In the second, established *T. annulata* schizont-infected cells activate naïve lymphocytes to proliferate [the *Theileria* mixed leucocyte reaction (MLR)] (Preston 1981; Campbell et al. 1995) as first described in cultures of naïve bovine lymphocytes incubated with *Theileria parva* schizont-infected cells (Pearson et al. 1979, 1982). Both $CD3^+$ T cells (Bussler et al. 1997) and $\gamma\delta$ T cells (Collins et al. 1996) proliferate under these conditions. The levels of proliferation attained in this reaction, which can be stimulated by autologous or allogeneic schizont-infected cells, are many times those seen when allogeneic uninfected bovine cells are incubated together. The stimulus for this non-specific proliferation is not yet clear. While it is assumed that a parasite-derived molecule is involved, this has still to be identified, but evidence exists that proliferation is linked to the production of interleukin (IL)-1α by schizont-infected cells (Brown et al. 1995).

The finding that *T. annulata* schizont-infected cells possess a powerful method of immune subversion raises the question as to how cattle, which recover from infection, manage to counteract this subversion and eliminate the parasite. The study reported here was carried out to see if the answer lay in the preliminary observation that macrophages harvested from cattle undergoing resolving infections not only inhibited proliferation of *T. annulata* schizont-infected cells but also suppressed the non-specific activation of lymphocytes by autologous schizont-infected cells (Preston 1981). Moderation of MLRs induced with *T. parva* schizont-infected cells by monocytes (Goddeeris and Morrison 1987) supported this proposition. The possibility that 'suppressor' macrophages may prevent the induction of non-specific lymphoproliferation by schizont-infected cells and so protect cattle from lethal disease was examined in vitro in the *Theileria* MLR using cells from calves belonging to different cattle breeds, which previous, as yet unpublished, work indicated would respond differently to inoculation with a high dose of *T. annulata* sporozoites. The *Bos indicus* (Sahiwal) calves were predicted to resist infection; the *Bos taurus* (Friesian) calves were unlikely to resist infection. In previous studies (Campbell et al. 1995; Brown et al. 1995) MLRs were stimulated by in vitro-derived schizont-infected cell lines prepared in culture by incubating sporozoites with peripheral blood mononuclear cells harvested from naïve calves. In an attempt to replicate in vivo conditions as closely as possible, the MLRs described below were carried out with ex vivo-derived lines prepared from cells harvested from infected cattle.

The work began by confirming that ex vivo-derived lines stimulate MLRs, defining the properties of schizont-infected Sahiwal cells and showing that a MLR carried out with Sahiwal cells resembled a MLR carried out with taurine cells. The influence of macrophages on lymphoproliferation was first examined in MLRs with cells harvested from naïve Sahiwal or naïve Friesian calves and then in MLRs with cells harvested during the course of infections initiated with a high dose of sporozoites of the *T. annulata* Hisar (Indian) stock. In the absence of any information on suppressor macrophages in tropical theileriosis, the choice of likely mediators of suppression was based on substances (nitric oxide, prostaglandin) shown to mediate suppression by macrophages in other parasitic infections (Allen et al. 1996). While the results obtained from the Sahiwal calves confirmed that suppressor macrophages can regulate lymphoproliferation and promote healing infections, the results obtained from the taurine calves suggested that unregulated activity of suppressor mechanisms could interfere with protective responses and promote overwhelming infection and disease. The existence of suppressor immune systems in infections with *T. annulata* has serious implications for the choice of candidate antigens in vaccine development. The last part of the work, therefore, examined lymphocyte-macrophage interactions in a group of cattle raised in an endemic area of tropical theileriosis, immunised with the *T. annulata* sporozoite antigen, SPAG-1, and the attenuated *T. annulata* Batan 2 cell line and then challenged with the virulent Tunisian Jed 4 stock.

Materials and methods

Reagents

RPMI-1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentimicin and 2 mM L-glutamine was used with 10% myoclone plus foetal calf serum (FCS) to maintain cell lines and with 20% FCS plus 10×10^{-5} M mercaptoethanol (Sigma Chemicals Ltd., Poole, Dorset, UK) for experiments [all ingredients from Gibco-BRL, Paisley, UK (now Invitrogen Ltd.)]. Myoclone plus FCS was used to avoid problems of toxicity for cell lines. Peripheral blood mononuclear cells (PBM) were prepared with Ficoll-Paque (Gibco-BRL). N^G-monomethyl-L-arginine (L-NMMA) and N^G-monomethyl-D-arginine (D-NMMA) were obtained from Alexis Corporation (Bingham, Nottingham, UK); indomethacin (I7378), mitomycin C (M4237) and phytohaemagglutinin (PHA) (L9132) from Sigma. All additives were diluted, aliquoted and stored at –60°C until needed. Methyl-^{3}H-thymidine (TRA 120) (tritium) was obtained from Amersham Life Sciences, Amersham, UK.

Cattle

PBM were obtained from three *B. indicus* (Sahiwal) calves (S82, S84, S85) bred at the Centre for Tropical Veterinary Medicine (CTVM) and three taurine (Friesian) calves (F78, F79, F80). All six

calves were inoculated sub-cutaneously in the subscapular region with a stabilated dose (0.1 tick equivalents (t.e.)) of *T. annulata* Hisar sporozoites shown previously to induce lethal infections in taurine calves. Sporozoite suspensions for the infection of cattle were prepared as described by Brown (1987). PBM were also obtained from another Sahiwal Calf (488) bred at the CTVM and a taurine calf (9). The following 12 taurine (Friesian-Holstein) calves, obtained from a modern state run theileriosis-free farm, were used as sources of PBM at l'Ecole Nationale de Medicine Veterinaire Sidi Thabet (ENMV): four normal (untreated) calves (calves 1–4); two calves (9, 77) undergoing severe infections on day 18–19, after inoculation with a lethal dose (0.5 t.e.) of sporozoites of the *T. annulata* (Jed 4) stock; four calves (52, 62, 71, 83) undergoing a challenge infection with a lethal dose of sporozoites of the Jed 4 stock (0.5 t.e.) – these calves had been immunised with a recombinant *T. annulata* sporozoite antigen, SPAG-1, – expressed as a fusion protein with a 6× Histidine tag as described by Hall et al. (2000) (His_6-SPAG-1) – in Montanide ISA 50 (Seppic) together with 2×10^6 cells of the attenuated *T. annulata* Batan 2 schizont-infected cell line (at passage 100); two 'carrier' calves (7, 38) which had recovered from a previous challenge with sporozoites of the Jedeida 4 stock after immunisation with the attenuated Ta Batan 2 cell line alone (calf 7) or with His_6-SPAG-1 in Montanide ISA 50 (calf 38). The two carrier calves and the immunised/challenged calves were treated with buparvaquone (2.5 mg/kg) on day 35 of the challenge infection to ensure that all parasites had been eliminated before the animals' cells were retested approximately 8 weeks later. The Hisar stock was described by Gill et al. (1976), the Batan 2 and Jed 4 stocks (as CL2 and CL4, respectively) by Darghouth et al. (1996). All calves were maintained throughout on commercial food and hay and water ab libitum. Their condition was assessed by monitoring the pattern of pyrexia (defined as rectal temperature over 39.5°C), haematological parameters such as leucopaenia and anaemia, parasitosis (schizont- and merozoite-infected cells) and parasitaemia (percentage erythrocytes infected with piroplasms) as described previously (Preston et al. 1992; Darghouth et al. 1996). Calves at ENMV were maintained in a tick-free building.

Cell lines

The methods for establishment of ex vivo-derived schizont-infected cell lines (cell lines) from PBM harvested from infected cattle, maintenance and subculture of cell lines were described by Brown (1987). *T. annulata* (Hisar) in ex vivo-derived cell lines prepared from Sahiwal calves 488 and 489 (Ta His 488, Ta His 489) and from three taurine calves (Ta His 10, Ta His12, Ta His 14) were used at the University of Edinburgh. *T. annulata* (Batan 2) in an ex vivo-derived cell line prepared from a naturally infected case of theileriosis (Ta Batan 2) (Darghouth et al. 1996) was used at ENMV.

Lymphoproliferation assays

The assay was based on that described previously by (Pearson et al. 1979; 1982). Cultures of PBM (i.e. lymphocytes plus macrophages) or cultures of non-adherent cells (NAC) (i.e. lymphocytes alone) were incubated in the presence of schizont-infected cells, PHA or medium for 5 days. Replicates of PBM plus stimulators (cell lines or PHA or medium alone) were also set up with indomethacin or L-NMMA or D-NMMA to see if either indomethacin or L-NMMA, as potential inhibitors of macrophage activity, influenced lymphoproliferation. D-NMMA served as a negative (inactive) control for the potential toxicity of L-NMMA. PBM were described as prepared by Preston et al. (1993). NAC were prepared by incubating PBM at a concentration of 20×10^5 cells/ml in 20% FCS/RPMI in flasks for 2 h at 37°C, removing the suspension of NAC and pipetting aliquots directly into the plates. Cell lines were inactivated by incubation with mitomycin C as *T. annulata* schizont-infected cell lines are very resistant to irradiation. 1×10^7 cells were incubated in 1 ml of mitomycin C (50 μg/ml in 20% FCS/RPMI) for 30 min at 37°C; washed twice in 10 ml 20% FCS/RPMI prior to resuspension at 20×10^4/ml in RPMI.

Cultures were set up as follows: 100-μl aliquots of PBM or NAC (20×10^5 cells/ml) in 20% FCS/RPMI with either 100 μl of mitomycin-C-treated schizont-infected cells (20×10^4 cells/ml) in RPMI, or with 100 μl PHA (1 μg/ml) in RPMI, or with 100 μl RPMI alone. In some experiments, additional sets of cultures were set up for incubation with additives: 20 μl of indomethacin (15 μg/ml) was added to one set; 20 μl of L-NMMA (0.5 mM) to another set and 20 μl D-NMMA (0.5 mM) to the third set. Each combination of cells plus stimulant was set up in quadruplicate. Experiments were carried out in a final concentration of 10% FCS/RPMI (5×10^{-5} M mercaptoethanol). Cultures were incubated for 5 days, unless mentioned otherwise in the text.

Proliferation in cultures set up at the University of Edinburgh (EU) was assessed by monitoring the incorporation of tritium (0.5 μCi ^{3}H-methyl-thymidine/well) (1.8 Bq/mmol) by the proliferating responder populations of cells over the last 18 h of the culture period. Culture plates were harvested with a Tomtec Harvester 96 (Mach III M); the incorporation of tritium by proliferating cells was measured using a Wallac Trilux 1450 Microbeta counter and expressed as counts per minute.

The proliferative response in cultures set up at ENMV was assessed on day 5 by the Cell Titer 96^R AQ_{ueous} Non-Radioactive Proliferation Assay (Promega Product G1112) using the manufacturer's instructions. This colorimetric method determines the number of viable cells in the culture via the bioreduction of a tetrazolium compound into a formazan product that is soluble in tissue culture medium. The quantity of this product is directly proportional to the number of living cells in culture. In brief, the cultures which had been incubated in U-bottomed well plates were transfered to flat-bottomed well plates for measuring absorbance on the ELISA reader.100 μl of phenazine methosulphate solution was added to each 2 ml of Owen's reagent (MTS) just prior to use and 40 μl of the mixture was added to each (200 μl) culture. The plates were incubated for 3 h in an humidified 5% CO_2 incubator at 37°C while the tetrazolium compound was bioreduced into the formazan product. The reaction was stopped by the addition of 50 μl 10% sodium dodecyl sulphate to each 200 μl well. The quantity of formazan product produced by each replicate culture was then measured by absorbance at 490 nM on an ELISA reader and expressed as optical density (OD). The results were calculated as follows: response in MLR = OD of cells in presence of schizont-infected cells minus OD of cells incubated in medium alone; response to PHA = OD of cells in presence of PHA minus OD of cells in presence of medium alone.

The following MLRs were carried out:

1. The PBM of Sahiwal calves 488 and 489 were tested with their autologous cell lines (Ta His 488, Ta His 489); the PBM of calf 9 with the Ta His 10 cell line at EU. Calves 9 and 10 were split-embryo twins and so their cells and cell lines were considered autologous.
2. The PBM and NAC of three Sahiwal calves (S82, S84, S85) and three Friesian calves (F78, F79, F80) were tested at EU with the Ta His 488 cell line or PHA before infection and at weekly intervals afterwards. Cultures of PBM were also incubated with indomethacin, L-NMMA or D-NMMA.
3. PBM and NAC from calves at the ENMV were tested with the Ta Batan 2 cell line or PHA at times given in the text. Cultures of PBM were incubated with indomethacin, L-NMMA or D-NMMA.
4. The cells of the two 'carrier' calves and the immunised/challenged calves tested at ENMV were retested 12 weeks later – 8 weeks after drug treatment – with the Ta Batan 2 cell line or PHA.

Phenotypic analysis of cell lines

The phenotypic profiles of the Ta His 488 and Ta His 489 cell lines were compared with the profiles of the three taurine cell lines (Ta His 10, Ta His 12, Ta His 14). The PBM of Sahiwal calf 488 and the

taurine calf 9 were phenotyped before and after incubation in MLRs. PBM and schizont-infected cell lines were phenotyped as described by Preston et al. (1992) and Forsyth et al. (1997) respectively using the panel of monoclonal antibodies to bovine leucocyte antigens described by Forsyth et al. (1997) but using sheep anti-mouse fluorescein-labelled IgG F(ab)' (Nordic) instead of sheep anti-mouse fluorescein-labelled gamma-globulin.

Assessment of cytokine mRNA expression by reverse transcription-polymerase chain reaction

The two Sahiwal cell lines, Ta His 488 and Ta His 489, and three cell taurine lines, Ta His 10, Ta His 12 and Ta His 14, were assessed for the mRNA expression of the following cytokines: IL-1α, IL-1β, IL-6, IL-10, IL-12, tumour necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ), by reverse transcription-polymerase chain reaction (RT-PCR) as described by Brown et al. (1995).

Analysis of results

A problem, discussed by Preston et al. (1992), in studying the immune responses of cattle is marked day to day variation in the responses of individual animals, in particular in MLRs. For this reason the overall responses of the cattle have been pooled to give a representative view of the results; the results of some groups of animals are presented as stimulation indices to show the individual responses obtained. The derivation of the stimulation indices is given in the text.

Results

Characteristics of schizont-infected cells of Sahiwal and taurine calves and assessment of their performance in the *Theileria* MLR

T. annulata schizont-infected cells derived from Sahiwal and taurine calves have similar but distinct host cell phenotypes

The phenotypic profiles of the two cell lines (Ta His 488, Ta His 489) prepared from schizont-infected cells harvested from Sahiwal calves 488 and 489 were very similar (Table 1) but differed from the profiles of the three cell lines prepared from cells harvested from infected taurine calves. Uniquely, the Sahiwal cell lines strongly expressed CD2 (27–54%) and CD8 (25–27%) (Table 1); neither of these antigens were expressed by the taurine cell lines. Otherwise all five lines expressed similar phenotypic profiles. As the phenotypes of the three taurine cell lines were identical only one is illustrated here (Table 1). The dominant antigens expressed by the Sahiwal lines were the myeloid markers TC17 and CD9, class II antigens, CD11b and CD49b. The dominant antigens on the taurine cell lines were also TC17 (76%), CD9 (30–42%), class II antigens (44–60%), CD11b (83–89%) and CD49b (57–75%). The mRNA cytokine profiles of both Sahiwal and taurine cell lines were similar in that they all produced mRNA for IL-1α, IL-1β, IL-6, TNF-α, but the Sahiwal cell lines also produced mRNA for IFN-γ. None of the cell lines produced mRNA for IL-10 or IL-12 (Fig. 1).

The MLRs initiated with Sahiwal PBM and schizont-infected Sahiwal cells resembled MLRs initiated with taurine PBM and schizont-infected taurine cells

The pattern of lymphoproliferation of PBM from Sahiwal calves 488 and 489 incubated with schizont-infected autologous cells are illustrated here by the MLR performed with the cells of Sahiwal calf 488. The results showed that the Sahiwal lymphocytes and infected cells interacted in a typical MLR (Fig. 2a). The timing of the response differed slightly from that of taurine calf 9 (Fig. 2b). However phenotypic analysis of the PBM of calves 9 and 488 prior to incubation and after 7 days of incubation showed that similar cell types had proliferated in both sets of MLR, namely $CD2^+$ T cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells and $\gamma\delta$ T cells (Fig. 2c, d). Cells bearing TC17 (myeloid markers) and BoWC1 (B cells) also multiplied in the MLR with Sahiwal cells but not in the MLR with taurine cells. These results indicated that PBM from the two cattle breeds could be used in MLRs with the Sahiwal Ta His 488 cell line to analysis the effect of macrophages on the non-specific proliferation of lymphocytes induced by schizont-infected cells.

Assessment of the effect of macrophages on the lymphoproliferative responses of Sahiwal calves undergoing healing infections with *T. annulata*

The clinical, haematolological and parasitological responses of the Sahiwal calves as they underwent healing infections are shown in Table 2.

Macrophages modulate lymphoproliferative responses in cultures of Sahiwal cells incubated with the Ta His 488 cell line

The levels of proliferation of cultures of whole PBM and NAC prepared from the Sahiwal calves prior to infection and incubated with Ta His 488 cells were very similar (Fig. 3a; Table 3, a). Comparison of the levels of proliferation of cultures prepared from calves after infection showed that the response of cultures of NAC alone was generally higher than the responses seen in cultures of PBM (Fig. 3a; Table 3, a). The reduced activity in cultures of PBM was sustained in two of the three calves (S84; S85) (Table 3, a). Incubation of cultures of PBM with indomethacin enhanced the lymphoproliferative response of PBM harvested from all three calves prior to infection (Fig. 3a; Table 3, b) and the responses of PBM harvested after infection (Fig. 3a). Enhancement was marked and sustained in cultures of PBM harvested from two of the three calves (S84, S85) (Table 3, b). Incubation of cultures of PBM with L-NMMA had no effect on the proliferative response (data not shown).

Table 1 Phenotypic analysis of two ex vivo-derived *Theileria annulata* (Hisar) schizont-infected Sahiwal cell lines (Ta His 488; Ta His 489) and an ex vivo-derived taurine cell line (Ta His 10) with a panel of monoclonal antibodies recognising bovine leucocyte antigens[a]. *ALVC* Afferent lymph veiled cells

Mabs	Antigen/cell lineage	Percentage cells reactive		
		Sahiwal lines		Friesian line
		Ta His 488	Ta His 489	Ta His 10
Medium alone	–	0.42 ± 0.07	0.17 ± 0.02	0.17 ± 0.02
Medium F(Ab)'	–	0.58 ± 0.01	0.27 ± 0.04	0.16 ± 0.06
Lymphoid markers				
CC42	CD2	53.9 ± 12.63	27.3 ± 0.64	4.08 ± 0.94
MMIA	CD3	0.74 ± 0.07	0.36 ± 0.06	0.19 ± 0.05
CC30	CD4	1.56 ± 0.46	6.12 ± 0.64	1.61 ± 0.49
CC63	CD8	25.54 ± 1.20	27.21 ± 1.42	1.39 ± 0.03
CACT61A	TCR1 ($\gamma\delta$ T cells)	0.78 ± 0.12	1.17 ± 0.56	0.25 ± 0.03
CC51	BoWC1	2.57 ± 0.56	2.25 ± 0.34	2.54 ± 0.15
Myeloid markers				
IL-A25	Myeloid cells[b]	1.76 ± 0.29	1.43 ± 0.75	0.41 ± 0.25
IL-A106	Monocytes/ALVC	3.23 ± 0.51	2.34 ± 0.16	4.72 ± 0.63
IL-A109	Monocytes[c]	1.64 ± 0.17	1.77 ± 0.27	3.32 ± 0.46
IL-A24	Myeloid cells (TC17)	63.07 ± 4.55	76.97 ± 0.77	75.95 ± 1.62
IL-A96	Myeloid cells (CD9)	28.60 ± 3.73	15.56 ± 0.94	42.59 ± 1.58
MHC antigens/adhesion antigens/complement receptor				
IL-A21	MHC II Ags	11.08 ± 2.11	23.41 ± 1.66	47.77 ± 0.47
IL-A15	CD11b	87.17 ± 3.34	88.72 ± 1.66	89.62 ± 1.66
Clone 218	CD49b	81.99 ± 0.62	71.03 ± 1.24	64.28 ± 1.74
Du-1-29	CD62L	0.66 ± 0.07	0.397 ± 0.16	0.19 ± 0.09

[a] Triplicate samples of cells were stained with each MAb; results expressed as percentage of reactive cells

[b] Pulmonary macrophages (MacHugh, personal communication)

[c] Putative CD64 (MacHugh et al. 1990)

Macrophages modulate lymphoproliferative response in cultures of Sahiwal cells incubated with PHA

The lymphoproliferative response of cultures of PBM and NAC harvested prior to infection differed: the response of the PBM being greater than the response of the NAC (Fig. 3b; Table 3 ,a). Comparison of the levels of proliferation of cultures prepared from calves after infection showed that the responses of cultures of NAC alone were higher than the responses seen in cultures of PBM (Fig. 3b; Table 3, a), except for the responses of two calves (S82, S84) on day 21 after infection. Incubation of cultures of PBM with indomethacin enhanced the lymphoproliferative response of PBM harvested prior to and after infection (Fig. 3b). This enhancement was seen in cultures prepared from all three calves except for PBM harvested on day 8 from two of the calves (S83, S85) (Table 3, b). Incubation of cultures with L-NMMA had no effect on the proliferative response (data not shown).

Assessment of the effect of macrophages on the lymphoproliferative responses of Friesian calves undergoing severe disease with *T. annulata*

The clinical, haematological and parasitological responses of the Friesian calves as they underwent severe disease are shown in Table 2.

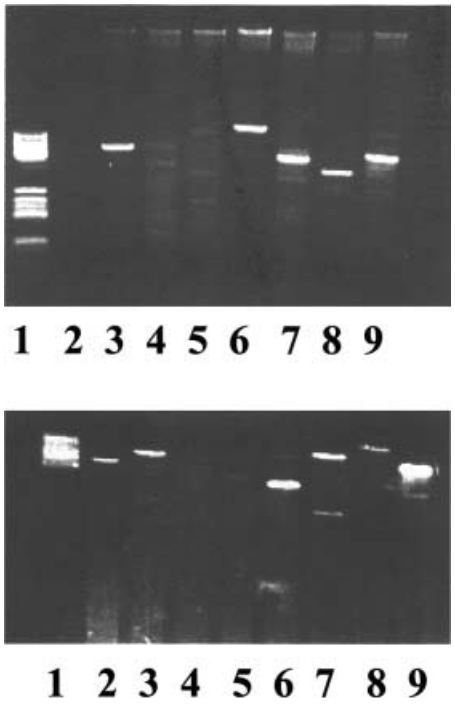

Fig. 1 Expression of mRNA for cytokines: by the taurine Ta His 9 cell line (*top*) and the Sahiwal Ta His 488 cell line (*bottom*) detected by reverse-transcription polymerase chain reaction analysis. From *left* to *right* the lanes held Hae III markers (1), primers for IFN-γ (2), TNF-α (3), IL-12 (4), IL-10 (5), IL-6 (6), IL-1β (7), IL-1α (8) and glucose 3-phosphate dehydrogenase (9)

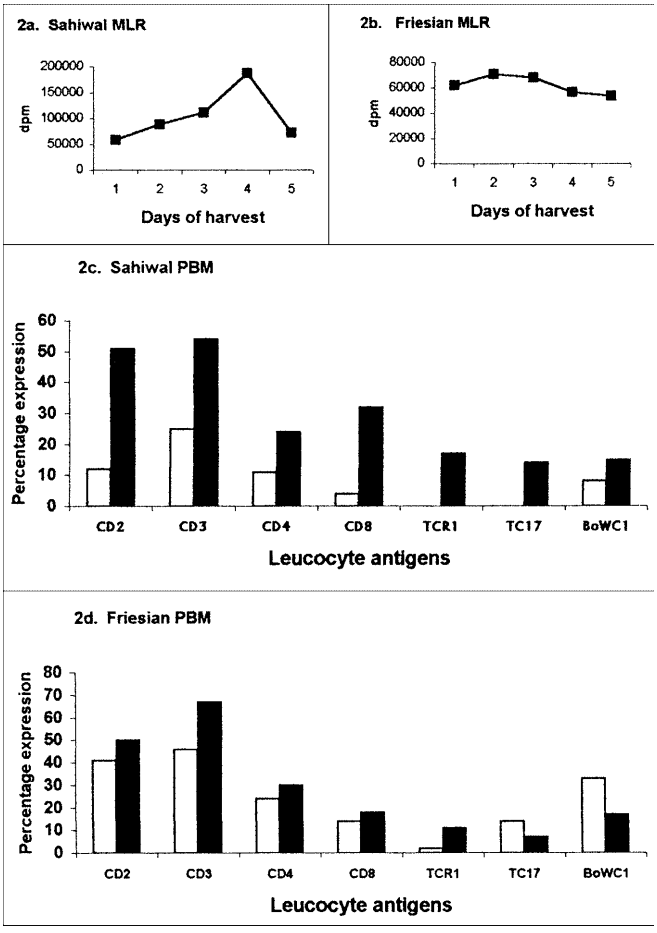

Fig. 2 Autologous '*Theileria*' mixed leucocyte reactions (MLRs): performed with **a** peripheral blood mononuclear cells (PBM) from Sahiwal calf 488 incubated with its 'autologous' Ta His 488 cell line; **b** PBM from Friesian calf 9 incubated with an 'autologous' Ta His10 cell line prepared from its infected syngeneic twin calf 10. Days 1–5 of harvest equate with days 4–8 of incubation. Lymphoproliferative responses expressed as disintegrations per minute (dpm). Phenotypic profile of PBM before and 7 days after incubation with autologous cells: **c** PBM from Sahiwal calf 488, **d** PBM from taurine calf 9. Results expressed as percentage of cells expressing the following leucocyte antigens: CD2, CD3, CD4, CD8, TCR1 (a $\gamma\delta$ T-cell marker); TC17 (a monocyte/macrophage marker); BoWC1 (a B-cell marker) before (*open squares*) and after (*filled squares*) incubation

Profound suppression of lymphoproliferative responses in cultures of Friesian cells incubated with the Ta His 488 cell line

The lymphoproliferative response of cultures of PBM and NAC harvested prior to infection differed. The response of the PBM being much greater than the response of the NAC (Fig. 4a; Table 4, a). The responses of NAC prepared from calves after infection were always higher than the responses of PBM, with the exception of the PBM of one calf (F80) on day 8 (Fig. 4a; Table 4, a). Incubation of cultures of PBM with indomethacin only slightly enhanced the lymphoproliferative response of PBM harvested before infection, had no effect on PBM harvested on days 8 and 15 after infection, but caused a slight enhancement on day 21 after infection (Fig. 4a; Table 4, b). Incubation of cultures with L-NMMA had no effect on the proliferative response (data not shown).

Profound suppression of lymphoproliferative response in cultures of Friesian cells incubated with PHA

The lymphoproliferative response of cultures of PBM and NAC harvested from the Friesian calves prior to infection were very variable (Table 4 ,a). The response of the PBM of one calf (F78) was lower than the response of its NAC; the responses of the PBM prepared from the two other calves (F79, F80) were greater than the responses of their NAC (Fig. 4b; Table 4, a). However, the responses of NAC prepared from all three calves after infection were always higher than the responses of PBM (Fig. 4b; Table 4, a). Incubation of cultures of PBM with indomethacin enhanced the lymphoproliferative response of PBM harvested before infection and on day 8 after infection but had no effect on PBM harvested on day 15 after infection; some enhancement of responses were seen in PBM harvested on day 21 after infection from two of the calves (F78, F80) (Fig. 4b; Table 4, b). Incubation of cultures with L-NMMA had no effect on the proliferative response (data not shown).

Assessment of the effect of macrophages on the lymphoproliferative responses of normal calves, of calves undergoing severe primary infections or challenge infections, after immunisation with His_6-SPAG-1 and the attenuated Ta Batan 2 cell line, and of carrier calves

PBM were harvested from the control calves (9, 77) on days 18–19 after infection when they were showing the typical signs of severe tropical theileriosis as recorded in the Friesian calves (F78, F79, F80). When PBM were harvested from the immunised calves they were in the process of resisting the challenge dose of sporozoites administered 18–19 days previously. The carrier calves appeared in good health. All the calves whose PBM were retested 2 months after treatment appeared in good health.

Profound suppression of lymphoproliferative responses in cultures of cells from immunised/challenged calves incubated with the Ta Batan 2 cell line

Cultures of PBM and NAC from normal (uninfected) and control calves undergoing a severe lethal infection responded similarly to incubation with the Batan 2 line (Fig. 5a). The response of NAC from calves undergoing challenge infections with a potentially lethal dose of sporozoites of the Jed 4 stock, after immunisation with

Table 2 Clinical, haematological and parasitological responses of Sahiwal and Friesian calves infected with 0.1 t.e. sporozoites of the T. *annulata* Hisar stock and the extent of lymphoid hyperplasia in the lymph node draining the site of inoculation. *T* Temperature; *LN* lymph node; *PCV* packed cell volume; *WBCs* white blood cells; –, not observed

Clinical and haematological responses	Days to $T > 39.5°C$	Duration $T > 39.5°C$	Peak *T* (°C)	Maximum reduction (%) in WBCs	Maximum reduction (%) in PCV	Day to death[a]
Friesians						
F78	9	12	40.9	85	45	23
F79	9	10	40.3	62	56	21
F80	6	12	41.0	66	42	22
Sahiwals						
S82	–	–	–	42	24	–
S84	9	4	40.1	67	51	–
S85	9	3	40.0	53	40	–
Parasitological responses	Days to 1st schizont	Duration parasitosis (days)	Peak parasitosis[b]	Duration merozoites (days)	Days to 1st piroplasm	Peak (%) parasitaemia
Friesians						
F78	8	16	+ + +	–	12	14
F79	5	15	+ + +	12–19	12	22
F80	6	10	+ + +	12-end	12	25
Sahiwals						
S82	8	5	+	–	12	2
S84	8	5	+ +	–	9	16
S85	7	3	+ +	–	9	10
Lymphoid hyperplasia[c]	Peak LN enlargement	Days to hyperplasia	Duration hyperplasia (days)	Maximum hyperplasia[d]	Maximum hyperplasia (days)	
Friesians						
F78	X10	8	19	+ +	9	
F79	X10	9	17	+ + +	11	
F80	X10	5	17	+ +	17	
Sahiwals						
S82	X4	7	12	+ +	6	
S84	X4	7	12	+ +	10	
S85	X4	9	10	+ + +	1	

[a] Calves were killed humanely by lethal injection when deterioration of clinical condition was accompanied by high parasitosis or parasitaemia and severely depressed haematological parameters

[b] +, <1%, + + +, >5%

[c] Presence of uninfected lymphoblastoid cells

[d] + +, moderate; + + +, extensive numbers of lymphoblastoid cells

His_6-SPAG-1 and an attenuated cell line, was markedly less than the response of NAC from either the uninfected calves or the control group of infected calves. The response of PBM from the immunised/challenged group was dramatically suppressed, so much so that the cells in these cultures appeared to be no longer metabolising. In spite of this suppression the calves recovered from the challenge infection and when their cells were tested several months later, their NAC responded as well as NAC from uninfected animals although their PBM still showed some slight suppressive activity. Neither indomethacin nor L-NMMA had any effect on lymphoproliferation.

Profound suppression of lymphoproliferative responses in cultures of cells from immunised/ challenged calves incubated with PHA

Lymphoproliferation in cultures of NAC from normal and control calves was less than in the cultures of PBM from these animals (Fig. 5b). The proliferative responses of both the NAC and PBM from the immunised/challenged calves were markedly less than the response of cells from either the normal or control calves. It appeared that the cells in these cultures were no longer metabolising. When the cells were retested after the calves recovered from the challenge infection, the NAC responded to a greater extent than the NAC from uninfected animals. The PBM still expressed some suppressive activity but less than when the calves were reacting to the challenge infection. Neither indomethacin nor L-NMMA affected the lymphoproliferative responses.

Assessment of the effect of macrophages on the lymphoproliferative responses of two 'carrier' calves

The two 'carrier' calves had recovered from a challenge dose given 15 weeks previously after immunisation with the attenuated Ta Batan 2 line (calf 7) or with the HIS_6-SPAG-1 antigen in Montanide ISA 50

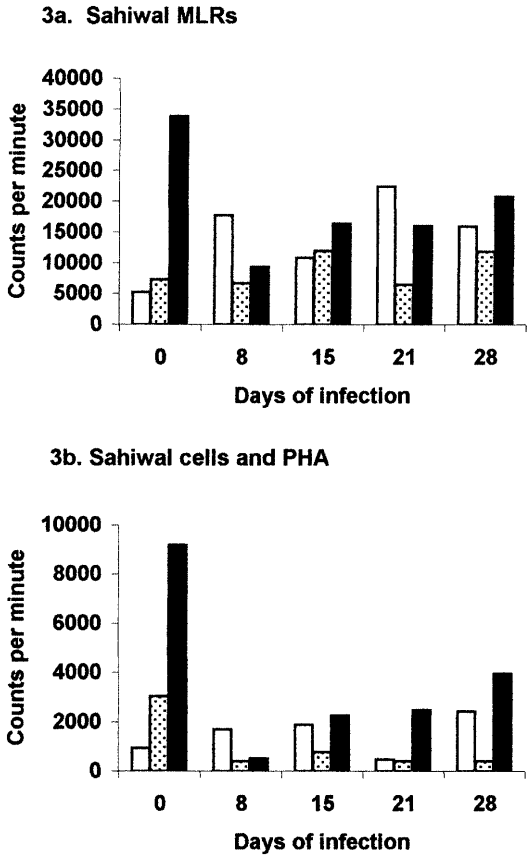

Fig. 3 **a** Autologous '*Theileria*' MLRs carried out with cells from the three Sahiwal calves: cells harvested before (day 0) and at intervals after infection with a 'lethal' dose of *T. annulata* (Hisar) sporozoites and incubated with the Ta His 488 cell line. **b** Proliferative responses to PHA of cells from the three Sahiwal calves: cells harvested before (day 0) and at intervals after infection. Lymphoproliferative responses of NAC (*open squares*); PBM (*squares with dots*); PBM incubated with indomethacin (*filled squares*) , expressed as counts per minute. Results represent the mean response of cultures from the three calves

(calf 38). The NAC of calf 7 responded to incubation with the Ta Batan 2 line, and the response was enhanced by inclusion of adherent cells in the cultures, i.e. its cells behaved as did cells from a normal animal (Fig. 6a). After drug treatment its NAC became more responsive but the adherent cells became suppressive. The NAC of calf 7 failed to respond to PHA before treatment; its PBM responded slightly (Fig. 6a); after treatment its NAC were responsive but its adherent cells were suppressive. The cells from the other 'carrier' calf (38) responded differently, both before and after treatment (Fig. 6b). Before treatment its NAC responded to the cell line and to PHA but responses to both were severely suppressed by the inclusion of adherent cells in the cultures, i.e. the cells behaved in the same way as cells from the immunised and challenged calves. After treatment, both NAC and PBM responded slightly to the Ta Batan 2 cell line, but the NAC did not respond to PHA and the PBM failed to metabolise at all.

Table 3 Responses of PBM and NAC harvested from *T. annulata*-infected Sahiwal calves, before infection (day 0) and at intervals after infection (days 8–28) and incubated with the Ta His 488 cell line or PHA. Results calculated to show (a) differences in lymphoproliferation in cultures of PBM and in cultures of NAC using a stimulation index derived by dividing the response of PBM by the response of NAC (cpm); (b) differences in lymphoproliferation in cultures of PBM incubated in medium alone and in cultures of PBM incubated with indomethacin using a stimulation index derived by dividing the response of PBM incubated with indomethacin by the response of PBM incubated in medium alone (cpm). Day: day after infection with *T. annulata* (Hisar) sporozoites

	Cells incubated with					
	Ta His 488 cell line			PHA		
(a) PBM/NAC						
Calves:	S82	S84	S85	S82	S84	S85
Day 0	1.35	2.16	1.01	2.64	2.89	4.55
Day 8	0.61	0.19	0.82	0.47	0.10	0.19
Day 15	1.56	0.41	0.98	0.43	0.37	0.60
Day 21	1.82	0.10	0.32	1.59	1.59	0.26
Day 28	1.35	0.61	0.47	0.26	0.07	0.14
(b) PBM + indomethacin/PBM						
Calves:	S82	S84	S85	S82	S84	S85
Day 0	5.11	2.78	5.34	2.61	5.31	2.73
Day 8	1.71	1.56	0.71	1.10	2.15	1.15
Day 15	1.06	3.28	1.36	2.89	2.56	2.14
Day 21	0.64	5.20	2.76	5.32	6.85	9.26
Day 28	0.96	2.48	2.15	6.64	33.05	3.78

Discussion

This study used the *Theileria* MLR to show that the non-specific proliferation of lymphocytes which characterises infections with *T. annulata* may be modulated by adherent cells. The inclusion of adherent cells in cultures of cells from naive Sahiwal and Friesian calves enhanced the non-specific lymphoproliferative response to schizont-infected cells. During infection, however, and in cultures of cells from both types of calves, the adherent cells became markedly suppressive, so that the lymphoproliferative responses of PBM were usually less than those of NAC. The suppression of lymphoproliferative responses to PHA by adherent cells showed that the immunosuppressive responses were of a generalised nature. The suppressor responses operating in cultures of Sahiwal cells incubated with schizont-infected cells or with PHA were susceptible to indomethacin indicating that the suppressor cells were macrophages. The suppressor activity operating in cultures of Friesian cells were not always modulated by indomethacin; the reasons for this are discussed below. The results provide the first detailed evidence that immunosuppressor macrophages can modulate the non-specific proliferative response of bovine lymphocytes to *T. annulata* schizont-infected cells via a prostaglandin-mediated pathway.

The failure of L-NMMA to modulate lymphoproliferation showed an apparent lack of a role for nitric oxide as a suppressor of the proliferative response of bovine lymphocytes. This was surprising in view of its

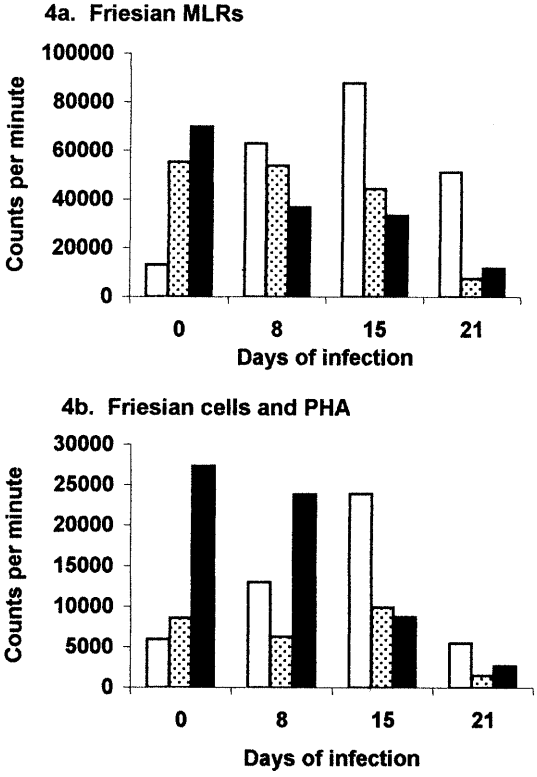

Fig. 4 **a** Autologous '*Theileria*' MLRs carried out with cells from the three Friesian calves: cells harvested before (day 0) and at intervals after infection with a 'lethal' dose of *T. annulata* (Hisar) sporozoites and incubated with the Ta His 488 cell line. **b** Proliferative responses to PHA of cells harvested from the three Friesian calves: before (day 0) and at intervals after infection. Lymphoproliferative response of NAC (*open squares*); PBM (*squares with dots*); PBM incubated with indomethacin (*filled squares*) expressed as counts per minute. Results represent the mean response of cultures from the three calves

Table 4 Response of PBM and NAC harvested from *T. annulata*-infected Friesian calves, before infection (day 0) and at intervals after infection (days 8–21), and incubated with the Ta His 488 cell line or PHA. Results calculated to show (a) differences in lymphoproliferation in cultures of PBM and in cultures of NAC using a stimulation index derived by dividing the response of PBM by the response of NAC (cpm); (b) differences in lymphoproliferation in cultures of PBM incubated in medium alone and in cultures of PBM incubated with indomethacin using a stimulation index derived by dividing the response of PBM incubated with indomethacin by the response of PBM incubated in medium alone (cpm). Day: day after infection with *T. annulata* (Hisar) sporozoites

	Cells incubated with					
	Ta His 488 cell line			PHA		
(a) PBM/NAC						
Calves:	F78	F79	F80	F78	F79	F80
Day 0	2.11	6.46	21.02	0.37	10.34	50.10
Day 8	0.55	0.51	2.0	0.57	0.20	0.52
Day 15	0.89	0.65	0.31	0.39	0.52	0.30
Day 21	0.24	0.09	0.05	0.33	0.13	1.08
(b) PBM + indomethacin/PBM						
Calves:	F78	F79	F80	F78	F79	F80
Day 0	1.3	1.37	1.17	3.36	4.20	2.63
Day 8	0.72	0.70	0.65	2.78	4.24	5.58
Day 15	0.62	0.85	0.98	0.91	1.02	0.69
Day 21	2.44	1.22	1.40	2.47	0.94	2.41

well documented role in the suppression of T-cell responses by suppressor macrophages in trypanosome infections (Sternberg and McGuigan 1992; Schliefer and Mansfield 1993; Mabbott et al. 1995). It could be that it has a role in suppression in theileriosis, but in synergy with prostaglandin, which would have been revealed if the two inhibitors (indomethacin and L-NMMA) had been included together in the cultures as in studies on trypanosomes. Attempts to see if suppression was mediated by reactive oxidative metabolites failed because the catalase inhibitor for these reactions proved toxic to the bovine cells at concentrations used in other systems (Allen et al. 1996).

There are several reasons why the suppressive cell activity in the Friesian calves sometimes failed to respond to indomethacin during the first 2 weeks of infection. Assuming the activity was due to a prostaglandin-mediated pathway, macrophages could have become temporarily refractory to regulation by indomethacin due to exposure to the excessively high levels of circulating IFN-γ, which are reported to occur during the first two weeks of heavy infection (Campbell et al. 1997). The renewed susceptibility to indomethacin of cultures prepared during the third week of infection supports this view. An alternative explanation is that other macrophage-mediated suppressor mechanisms became active as disease progressed and inhibition of macrophage activity required additional inhibitors as shown in murine models of trypanosome infection where prostaglandin and nitric oxide act synergistically (Schlieffer and Mansfield 1993). A third explanation is that other types of suppressor cells are involved in suppression. Whatever the explanation for the loss of activity, immunosuppressor macrophages acting via a prostaglandin-mediated pathway clearly occurred in Friesian calves undergoing severe disease. The question of whether additional mechanisms were active and, if so, their identity cannot be answered as yet. These results suggest that the low levels of non-specific immunosuppression recorded in cattle inoculated with *T. annulata*-schizont-infected cells (Sharpe and Langley 1983) could have been mediated by immunosuppressor macrophages.

The response of Friesian calves to the high dose of sporozoites used in this study resembled the responses of other Friesians inoculated with high doses of sporozoites (Preston et al. 1992; Forsyth et al. 1999). The ability of the Sahiwal calves to recover from doses of sporozoites which caused severe disease in Friesian calves is to be published in full elsewhere and confirmed earlier unpublished results which showed that another pair of Sahiwal calves (488, 489) recovered from infection with a dose of sporozoites which led to lethal disease in Friesian calves. The findings obtained by this study include a number of particular features which may be related to the differing responses of the two cattle breeds to a 'lethal' dose of sporozoites'.

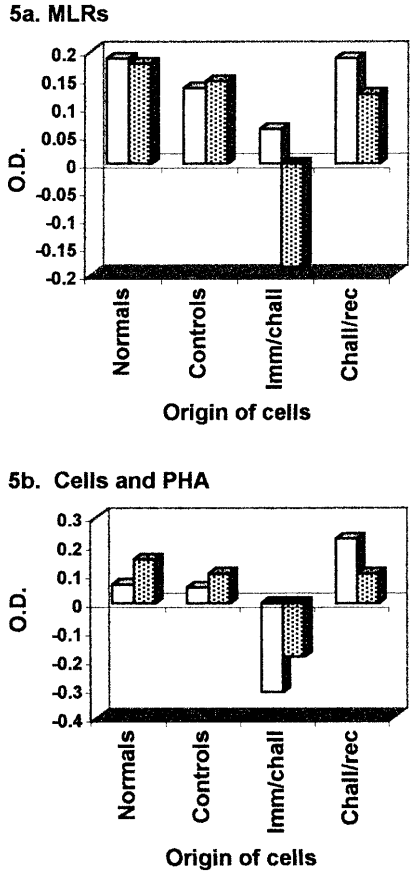

Fig. 5 **a** Autologous '*Theileria*' MLRs carried out by incubating cells from normal, infected or immunised/challenged Holstein-Friesian calves with the Ta Batan 2 cell line. **b** Proliferative responses to PHA of cells from the same groups of calves. Untreated calves (normals); infected calves undergoing severe disease (controls); immunised calves undergoing challenge infection (imm/chall); immunised calves recovered from challenge infection (chall/recov). Lymphoproliferative responses of NAC (*open squares*); PBM (*squares with dots*). Results represent the mean response of cultures from all the calves in each group, assessed by a non-radioactive, colorimetric method and measured as optical density (OD) units by an ELISA reader

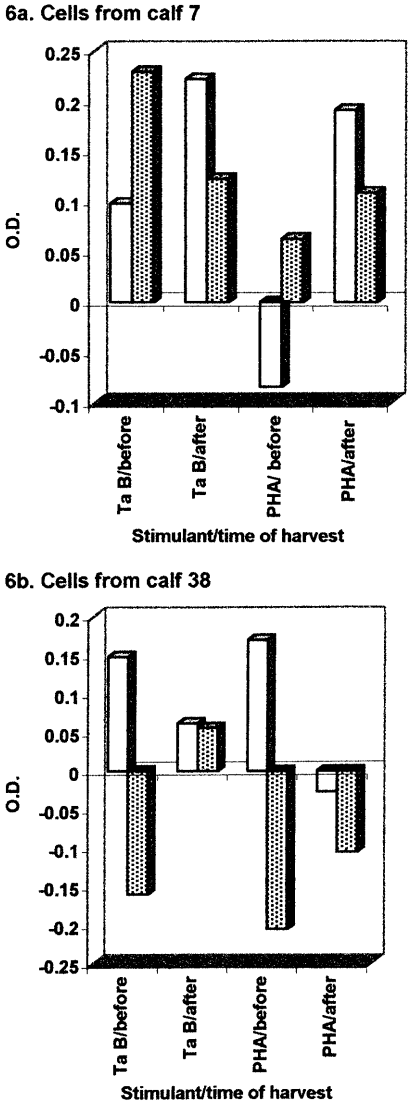

Fig. 6 Autologous '*Theileria*' MLRs carried out by incubating cells from two 'carrier' calves with the Ta Batan 2 cell line and lymphoproliferative responses of their cells to PHA: **a** cells from calf 7 immunised with the attenuated Ta Batan 2 cell line; **b** cells from calf 38 immunised with His_6-SPAG-1; both calves were challenged with sporozoites of Jed 4 stock. Lymphoproliferative responses of NAC (*open squares*); PBM (*squares with dots*). Cells tested before (before) and after (after) drug treatment. Results represent the mean response of cultures from all the calves in each group, assessed by a non-radioactive, colorimetric method and measured as optical density (OD) units by an ELISA reader

While the phenotypic profiles of the two Sahiwal cell lines and the three taurine cell lines were very similar and suggestive of a myeloid origin, the coexpression of CD2, CD8 and CD11b, in the absence of CD3 and CD4, by the Sahiwal lines suggested that some schizonts inhabited NK cells. The mRNA profiles of the ex vivo-derived taurine lines were similar to those of in vitro taurine lines analysed previously (Brown et al. 1995) indicating that the lines were macrophage in origin. The production of mRNA for IFN-γ by the Sahiwal cell lines provided additional evidence that at least some of these cells were NK cells. If schizont-infected Sahiwal cells secrete IFN-γ then they may trigger an early protective response in Sahiwal cattle by activating macrophages to produce nitric oxide and inhibit the proliferation of parasitised cells (Preston et al. 1999) thereby reducing parasite load during the initial stages of infection.

The overall levels of lymphoproliferation of cells from the Sahiwal calves (S82, S84, S85), both in cultures of NAC alone or of PBM, were consistently less than the levels of lymphoproliferation in equivalent cultures of cells prepared from the Friesian calves (F78, F79, F80). These findings suggested that the Sahiwal lymphocytes were intrinsically less susceptible to the induction of non-specific proliferation by schizont-infected cells than the Friesian lymphocytes. Using the same schizont-infected cell line (Ta His 488) to stimulate both the

Sahiwal and Friesian MLRs eliminated any variation in response which might have been due to the different cell types inhabited by schizonts in Sahiwal and Friesian cattle. The different levels of proliferation in the in vitro cultures generally reflected the different levels and duration of hyperplasia recorded in the lymph nodes draining the site of inoculation in the two cattle types (Table 2).

The third difference, which may have been crucial in determining the outcome of infection in the two different types of cattle, was the differing susceptibilities of the adherent cells of the two cattle breeds to indomethacin. The behaviour of adherent cells prepared from the Sahiwals appeared to be part of a well-regulated immune response where excessive lymphoproliferation was controlled by a prostaglandin-dependent macrophage mediated feedback mechanism as has been recorded in other conditions (Schlieffer and Mansfield 1995; Allen et al. 1996) and where the immunosuppressive macrophages also remained susceptible to regulation. For unknown reasons a more profound suppression of lymphoproliferation which became temporarily refractory to the prostaglandin inhibitor occurred in Friesian calves.

Together these findings suggest that Sahiwals recover successfully from infection because they express a balanced and integrated protective immune response which is probably acclerated by the triggering of an innate immune response (Preston et al. 1999) by IFN-γ derived from schizont-infected cells. The efficacy of the protective responses in the Sahiwal calves was shown by the reduced levels and duration of schizont and piroplasm populations, the reduced severity of pyrexic responses, leucopaenia to some extent and anaemia (as expressed by maximum reduction in PCV) as compared to Friesian calves (Table 2).

In contrast, the development of severe clinical disease in Friesian calves, as manifested by pyrexia, leucopaenia, anemia, prolonged and intense parasitosis, and high levels of parasitaemia (Table 2), was accompanied by prolonged and marked immunosuppressor mechanisms. In Friesians, the lack of IFN-γ production by schizont-infected cells may mean that an early innate response will not be triggered as rapidly as in Sahiwal calves and so schizont-infected cells will multiply more freely. It may be surmised that the initial stimulation of lymphocytes by schizont-infected cells triggers suppressor mechanisms which, if unregulated, also inhibit the antigen-dependent lymphocyte responses upon which elimination of the parasites and recovery from the disease depend (Preston et al. 1999), permitting uncontrolled parasite multiplication and promoting non-healing disease. Support for this hypothesis was provided by the following observations in taurine cattle inoculated with high doses of *T. annulata* sporozoites. The signs of an initial activation of lymphocytes in lymph nodes draining the site of inoculation of *T. annulata* sporozoites (Campbell et al. 1995) were followed by germinal cortical degeneration (Campbell et al. 1995), a widespread depletion of lymphocytes in the presence of an increasing histiocytic response throughout the lymphoid system (Forsyth 1997) and monocytosis in the peripheral blood (Preston et al. 1992). IFN-γ is well documented as a stimulator of prostaglandin synthesis (reviewed in Schleifer and Mansfield 1993) and is produced by lymphocytes non-specifically activated by schizont-infected cells (Ahmed et al. 1989; Campbell et al. 1997). The obvious candidate therefore for the activation of suppressor macrophages aimed at dampening down lymphoproliferation would be the unnaturally high levels of IFN-γ produced in cattle during the first 2 weeks after inoculation with a 'lethal' dose of sporozoites, as a consequence of the intense activation of lymphocytes by schizont-infected cells (Campbell et al. 1997).

Experiments carried out in calves raised in an endemic area of tropical theileriosis and infected with sporozoites of the virulent *T. annulata* Jed 4 stock provided evidence for the existence of an alternative suppressor mechanism. The proliferation of lymphocytes, prepared from normal calves and control animals undergoing lethal infections, and incubated with schizont-infected cells of the Ta Batan 2 stock was not apparently moderated by the inclusion of macrophages in the cultures. NAC prepared from normal and control infected animals responded to PHA; this mitogenic response was enhanced by the inclusion of adherent cells in cultures. However the metabolic levels of MLRs carried out with PBM prepared from calves undergoing challenge infections after immunisation were less than the metabolic levels of PBM incubated in medium alone. This finding indicated that the lymphocytes were not just suppressed in activity but had in fact been killed by the inclusion of adherent cells in the cultures. This cytotoxic effect disappeared after the calves had recovered from challenge; although adherent cells were still suppressive to some extent. The pattern of mitogenic responses of cells from the immunised and challenged animals showed that was a generalised immunosuppression. Failure to reverse any of these effects with either indomethacin or L-NMMA indicated that the adherent cells of these calves behaved differently from immunosuppressor macrophages obtained from the infected taurine and Sahiwal calves described above. The reasons for these differences may be that suppressor cell pathways in this type of calves depended upon a synergistic effect of mediators so that detection requires more than one inhibitor or that other suppressor mechanisms were activated by the immunisation and challenge procedures undergone by the animals.

The Tunisian, Friesian-Holstein, calves whose cells manifested a profound immunosuppresssion were linked in that they had all, at one time or another, been inoculated with His_6-SPAG-1 either alone (calf 38) or together with the attenuated Ta Batan 2 cell line (calves 52, 62, 71, 83). Their immunosuppresive responses were reminiscent of a profound suppressor activity observed in a previous vaccination trial with the SR1 determinant

of the SPAG-1 molecule (Boulter et al. 1995). Among the $CD4^+$ T cells generated after the immunised calves were challenged with sporozoites was a population of Th_2-$CD4^+$-IL-4-producing cells (Glass et al. 1994). The discovery in a murine model of filariasis (MacDonald et al. 1999) of IL-4 dependent macrophages, which suppress the proliferation of transformed cell lines and mitogenic responses by a contact-dependent mechanism, may provide the clue to the nature of the profound suppression described here in SPAG-1 immunised/challenged calves. Perhaps the profound suppressor activity in PBM prepared from the immunised/challenged calves was caused by suppressor macrophages activated by the IL-4 produced by the Th_2 $CD4^+$ T cells which were induced by challenging immunised calves with sporozoites, and not by prostaglandin-mediated suppressor macrophages which occurred in infected calves and appeared to be activated by IFN-γ derived from non-specifically activated Th_1 $CD4^+$ T cells. Failure to modulate the former type of bovine adherent cell suppressor activity by indomethacin or L-NMMA accords with the contact-dependence of the murine system (Macdonald et al. 1999). These observations raise the question as to whether the virulence of *T. annulata* stocks, like the Jed 4, is linked to expression of the suppressor epitope of the SPAG-1 antigen. In spite of the profound suppression manifested by cocultivation of T cells and macrophages in vitro, the immunised calves recovered from challenge indicating that the protective mechanisms responsible for controlling multiplication of schizont-infected cells were unaffected by this response.

Our current limited knowledge of *T. annulata* infections prevents our understanding the interactions that determine why some infections induce severe disease, accompanied by profound immunosuppression, disruption of immune responses and pronounced pathological damage to the immune system, and other infections only induce mild reactions, immunocompetent responses and slight pathological damage. What is clear is that *Theileria* infections stimulate immune responses that can suppress non-specific activation of lymphocyte proliferation. If these suppressor responses are limited to suppressing non-specific T-cell proliferation and the associated immune disruption, and then turned off, they may be envisaged as promoting recovery. If suppressor responses are unregulated and also inhibit protective lymphocyte responses, they may be envisaged as promoting disease. The final effect of suppressor responses will be determined no doubt by the intensity of their activation, the nature of their suppressive mechanism – whether due to a soluble factor or contact dependent – and/or by the extent of their influence – whether localised or widespread – in the body. The intensity and nature of activation may be of particular importance in determining whether the dominant effect of macrophage activity is suppression of T-cell proliferation or the successful expression of microbicidal activities, including those which control *T. annulata* (Preston and Brown 1988; Preston et al. 1999). An additional complicating issue in determining the outcome of infection will be the contribution of the various innate and adaptive responses, which are thought to cooperate to control infection (Preston et al. 1999), to the particular reaction of an individual. The importance of concentration, environment and interaction with other immune mediators in determining whether the effects of cytokines will be pro- or anti-inflammatory and whether parasitic infections will result in pathology and disease or protection has been discussed by Omer et al. (2000).

In sum, this study provided solid evidence for the existence of potent suppressor macrophages in infections with *T. annulata* and further evidence that suppressor mechanisms may be activated by sporozoite antigens. The circumstances under which these mechanisms operated appeared to be governed by cattle breed, parasite load, prior immunological experiences and possibly parasite stock. Under some circumstances, immunosuppressive mechanisms appeared to be helpful; under other circumstances they appeared to promote severe disease. Differences in the nature of the schizont-infected cells of Sahiwal and taurine calves and the behaviour of their immunosuppresive responses provided several reasons why the two cattle breeds differ in susceptibility to infection with *T. annulata*. In extending our knowledge of the pluripotential activities of macrophages in *T. annulata* infections to include immunosuppression as well as anti-parasite responses (Preston et al. 1999), these findings provide yet more evidence that the outcome of infection with *T. annulata*, like many other parasitic infections, 'hinges on a delicate balance between appropriate and inappropriate activities of the immune system and its mediators (Omer et al. 2000)'. The demonstration that suppressor mechanisms are an integral part of the immune response to *T. annulat*a adds an additional complication to attempts to identify candidate vaccine antigens.

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