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Theileria annulata and T. parva infect and transform different bovine mononuclear cells

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SUMMARY

Bovine peripheral blood mononuclear cells (PBMC) were labelled with monoclonal antibodies recognizing bovine MHC class II, sIgM, monocyte, T-helper and T-cytotoxic cell phenotypes. They were sorted into positive and negative populations with a fluorescence-activated cell sorter (FACS). The cell populations were infected in vitro with sporozoites of either Theileria annulata or T. parva, and the degree of infection and transformation determined. The results showed that despite the many similarities between these two parasites, they infected different cells of the immune system. T. annulata preferentially infected MHC class II-positive cells but did not infect T cells. Monocytes were infected very efficiently by T. annulata but were uninfectable with T. parva. B cells were infected much more efficiently by T. annulata than T. parva. Cell lines derived from infections with T. annulata were analysed phenotypically. Virtually all reactivity was lost for the anti-sIgM and the anti-monocyte monoclonal antibodies post-infection and no T-cell markers were detected.

INTRODUCTION

Tropical theileriosis is a disease of major importance throughout North Africa and Southern Asia. It is caused by the protozoan parasite Theileria annulata, which is transmitted to the bovine host by ticks of the genus Hyalomma. East Coast fever, which occurs in East and Central Africa, is caused by the related parasite T. parva and is transmitted only by the tick Rhipicephalus appendiculatus. In addition to the differences between these two parasites in their geographical distribution and vector ticks, there are also differences in pathogenesis: T. parva produces greater mortality than T. annulata. However, a feature unique to both parasites is their ability to infect and transform cells of the immune system both in vivo and in vitro to produce continuously growing lymphoblastoid cell lines.

Although there have been several studies to identify the cell types infected and transformed by T. parva, it is not known which cells are infected with T. annulata. Cell lines arising from infection with T. parva generally show markers characteristic of T cells and most express MHC class II antigens but not sIgM (Naessens et al., 1985; Lalor, Morrison & Black, 1986; Baldwin et al., 1988). We have shown recently that established T. annulata-infected lines never express T-cell markers or surface (s)IgM but do express major histocompatibility complex (MHC) class II antigens (Spooner et al., 1988).

It has been shown that T. parva infects T cells, B cells and null cells (Lalor et al., 1986; Morrison et al., 1986; Baldwin et al., 1988). It is not established with certainty whether T. parva infects macrophages. One report suggested that macrophages cultured for several days could be infected (Moulton et al., 1984), whereas others found no evidence of infection and transformation of fresh monocyte/macrophage populations (Morrison et al., 1986; Baldwin et al., 1988). In contrast preliminary evidence suggested that T. annulata infects adherent cells (Musime, 1983). It is not known whether T. annulata infects and transforms T cells. The above evidence suggests that the two parasites may infect different cells of the immune system, which may have relevance to the observed differences between the two diseases.

In this paper we report infection of purified subpopulations of bovine peripheral blood mononuclear cells (PBMC), including monocytes, B cells and T cells, with either T. annulata or T. parva sporozoites. The results show that the two parasites infect and transform different cell types.

MATERIALS AND METHODS

Cell preparation

Blood was collected aseptically in 20% v/v acid citrate dextrose (ACD) from Friesian cows. Peripheral blood mononuclear cells (PBMC) were separated on Ficoll-Hypaque (Lymphoprep, NYCOMED AS, Oslo, Norway). Viability was always greater than 95% as assessed using Trypan blue dye exclusion.
Sporozoite preparation

*Hyalomma a. anatolicum* ticks, previously infected with *T. annulata* Hissar or Ankara (Walker et al., 1985) and fed for 3 days on rabbits, were ground up in MEM (Gibco, Paisley, Renfrewshire) with 3-5% bovine plasma albumin (BPA) (Sigma, Poole, Dorset). The supernatant was filtered and harvested as ground up tick supernatant (GUTS). The *T. parva* sporozoites were prepared from previously infected *Rhipicephalus appendiculatus* ticks fed for 4 days on rabbits. Salivary glands were dissected into Liebowitz L15 (Gibco) and homogenized and subsequently diluted in MEM + 3-5% BPA. (Brown, 1987). The *T. annulata* GUTS filtrates were used at 0-5 tick equivalents/ml and the *T. parva* salivary gland homogenate at 10 tick equivalents/ml. This produced approximately equal levels of infectivity for the two parasites on unsorted PBMC. Each experiment utilized a fresh batch of ticks.

Monoclonal antibodies

Five monoclonal antibodies (mAbs) were used in this study; IL-A11 detects BoT4, the bovine equivalent of human T4 (Baldwin et al., 1986; Teale et al., 1986), IL-A17 detects BoT8, the bovine equivalent of human T8 (Ellis et al., 1986), B5/4 reacts with bovine slgM (Pinder et al., 1980) and IL-A24 reacts with monocytes and granulocytes (Ellis et al., 1987; Glass & Spooner, 1989). J11 reacts with bovine MHC class II (Baldwin, Morrison & Naesens, 1987). The mAbs used for preparing cells for FACS sorting were diluted 1:100 in RPMI-1640 with 25 mm HEPES and 10% fetal calf serum (FCS) (Gibco), dialysed against RPMI-1640 with 25 mm HEPES for 24 hr, and filter sterilized.

Immunofluorescent staining

For analysis. This was performed as described by Spooner et al. (1988).

For FACS sorting. Indirect membrane staining was performed in RPMI-1640 with 25 mm HEPES and 5% gamma globulin-free horse serum (HS) (Gibco). Two millilitres PBMC at 10⁶ cells/ml were mixed with an equal volume of diluted mAb at 4°C. The mAbs were used at dilutions which gave maximal staining. After 30 min incubation at 4°C the cells were washed three times in RPMI-1640 containing 25 mm HEPES, 5% HS, 100 IU/ml penicillin and 100 μg/ml streptomycin (sorting medium) and then 1 ml of a FITC-conjugated anti-mouse Ig (RAM Ig; Nordic, Maidenhead, Somerset) was added at a dilution of 1:40. After 30 min incubation at 4°C the cells were again washed three times in sorting medium and maintained at 4°C prior to sorting.

Cell sorting

Cells were sorted on a FACs IV fluorescence-activated cell sorter (Becton-Dickinson & Co., FACS Systems, Sunnyvale, CA). The calibration of the equipment was as described by Spooner et al. (1988). Cells were collected in sterile phosphate-buffered saline pH 7.2 (PBS) and samples of the negative and positive populations were re-analysed to estimate the efficiency of the separation. The sorted cells were washed once at 100 g and resuspended in RPMI-1640 with 25 mm HEPES, 20% FCS, 200 IU/ml penicillin and 200 μg/ml streptomycin.

Cell infections

**Fixed concentration (T. annulata only).** Subpopulations of PBMC enriched or depleted for T cells were produced by FAC sorting using a combination of mAbs IL-A11 (T4) and IL-A17 (T8). Populations depleted or enriched for MHC class II-positive cells were produced using mAb J11. The cells were adjusted to 2 × 10⁶ cells/ml. Equal volumes (100 μl of cell suspension and 100 μl *T. annulata* sporozoite suspension were incubated together in triplicate in 96-well flat-bottomed tissue culture plates in a 5% CO₂/air mixture at 38°C. At 4 days post-infection, cytospin samples were prepared (see below) and the percentage of infected cells estimated for each subpopulation.

**Limiting dilutions (T. annulata and T. parva separately).** Sorted cells at 10⁶/ml or lower, depending on availability, were mixed with equal volumes of the sporozoite preparation and incubated at 38°C for 1 hr. Log₂ dilutions of this mixture were then made in complete tissue culture medium (RPMI-1640 with 25 mm HEPES, 10% FCS, 2 mm L-glutamine with 100 IU/ml penicillin and 100 μg/ml streptomycin). Then 20 replicate cultures of 200 μl/well were set up in 96-well (round-bottomed) culture plates in log₂ dilution steps to 1 cell/well. In some cases, such as with the IL-A24-positive sort, fewer cells were available and the highest concentration was 10⁵ cells/well. Filler cells (PBMC exposed to 5000 rads of gamma irradiation) were added to each well for the *T. parva* infections at 5 × 10⁵ cells/well. No filler cells were used for the *T. annulata* infections. Transformation was assessed by the presence of blast cells at 4 days, using a Leitz Diavert phase-contrast microscope (Leitz, Wetzler, FRG). Transformed cells were at least four times larger than untransformed cells and could be seen easily without staining. The plates were then maintained in 5% CO₂/air mixture for a further 3 weeks. There was no significant change in the dilution to which transformation was seen between 4 days and 3 weeks. Thus only the Day 4 readings were used.

Transformed cells were removed from individual wells in the limiting dilution plates at 19 days post-infection. These were stained with mAbs and then analysed with the FACS as described by Spooner et al. (1988).

**Cytospins**

Cytocentrifuge smears (Cytospin, Shandon, Runcorn, Cheshire) were prepared from representative wells from the limiting-dilution experiments and flat wells. These were stained with Giemsa and examined with an Ortholux II microscope at 1250 × magnification (LEITZ). The percentage of parasite-infected cells was estimated from counting 200 cells.

**RESULTS**

**Infections with *T. annulata***

To determine which cell populations of PBMC could be infected with *T. annulata* sporozoites, subpopulations of PBMC were isolated using the FACS. T cells, non-T cells, MHC class II-positive and class II-negative cells were incubated with *T. annulata* sporozoites and the percentage of transformed infected cells was assessed. The results of two experiments with cells from different animals on different days are shown in Table 1. Here it can be seen that *T. annulata* infects class II-positive cells very efficiently and T cells very inefficiently.

**Infections with *T. annulata* and *T. parva***

The results of infection with *T. annulata* only showed that this parasite infected and transformed class II-positive cells very
Table 1. Infection of mononuclear cell subpopulations with *T. annulata*

<table>
<thead>
<tr>
<th>Animal providing PBMC</th>
<th>Animal 1</th>
<th>Animal 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subpopulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-cell enriched</td>
<td>8</td>
<td>&lt;10</td>
</tr>
<tr>
<td>T-cell depleted</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>MHC class II positive</td>
<td>81</td>
<td>90</td>
</tr>
<tr>
<td>MHC class II negative</td>
<td>10</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Figure 1. FACS profiles of PBMC stained with IL-A24 (a) showing a large negative and small (5%) positive population. (b), (c) and (d) show the scatter plots before and after sorting. The sort windows are shown and there is negligible contamination in the two sorted samples. The IL-A24-positive cells have a higher 90% scatter profile as expected with a monocyte population.

Figure 2. Infection and transformation with *T. annulata* and *T. parva*. (a) IL-A24-positive cells where the maximum cell concentration available was 100 cells/well, and (b) IL-A24-negative cells.

Table 2. FACS analysis of cell lines arising from infection of PBMC and sorted cells with *T. annulata* showing the percentage of cells positive for MHC class II, T4, T8, monocyte and sIgM antigens 19 days post-infection

<table>
<thead>
<tr>
<th>Antigen detected</th>
<th>PBMC pre-infection</th>
<th>PBMC</th>
<th>IgM</th>
<th>IL-A24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class II</td>
<td>20.2</td>
<td>95.7</td>
<td>82.6</td>
<td>85.8</td>
</tr>
<tr>
<td>T4</td>
<td>30.0</td>
<td>0.9</td>
<td>4.5</td>
<td>3.5</td>
</tr>
<tr>
<td>T8</td>
<td>33.7</td>
<td>0.7</td>
<td>5.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Monocytes</td>
<td>4.0</td>
<td>2.3</td>
<td>2.9</td>
<td>4.6</td>
</tr>
<tr>
<td>IgM</td>
<td>23.0</td>
<td>1.1</td>
<td>6.2</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Monocytes

PBMC were sorted on the FACS using the monocyte-specific mAb IL-A24. The initial percentage of IL-A24-positive cells in PBMC varied between 4% and 6% on different days (data not shown). The FACS profiles of the unsorted and positive and negative populations are shown in Fig. 1. The IL-A24-positive sample was contaminated with 1% negative cells and the negative sample with 4% positive cells. These sorted populations were then incubated with sporozoites of either *T. parva* or *T. annulata* and the degree of infection and transformation estimated. The *T. annulata* infection curve for the monocyte preparation (Fig. 2a) indicates that almost every IL-A24-positive cell was infected and transformed. In contrast *T. parva* did not infect any IL-A24-positive cells.

With the IL-A24-negative population (Fig. 2b) there was no difference in infectivity between *T. annulata* and *T. parva* sporozoites. In fact the infectivity of this population was indistinguishable from that of unsorted PBMC (data not shown).

B cells

The anti-sIgM mAb, B5/4, was used to prepare a purified population of B cells from PBMC. The proportion of sIgM-positive cells in the initial PBMC sample was 23% (Table 2).
**T. annulata and T. parva**

**DISCUSSION**

We have shown for the first time that *T. annulata* and *T. parva* infect different cells of the bovine immune system. Virtually 100% of monocytes and B cells are infected with *T. annulata*, whereas T cells are not infected. In contrast *T. parva* does not infect monocytes and infects B cells less efficiently than *T. annulata*. The evidence presented in this paper generally supports other recent studies on the cell types infected by *T. parva* (Baldwin et al., 1988). However, our results indicate that either *T. parva* infects B cells inefficiently or only infects a subpopulation. This would fit with the findings of Baldwin et al. (1988) that it is difficult to produce sIgM-positive lines following *T. parva* infection unless the cells are cloned immediately after infection.

All of the cell lines arising from infection with *T. annulata* have the same phenotype, regardless of the cell subpopulation infected. They are all MHC class II positive but lack all reactivity for T-cell markers, and by 19 days post-infection have very low levels of monocyte and B-cell markers. This agrees with data on 34 long-term *T. annulata*-infected cell lines, none of which expressed T-cell, B-cell or monocyte markers but were all class II positive (Spooner et al., 1988). This indicates that although the sporozoites clearly infected both monocytes and B cells, the IL-A24 marker and sIgM are rapidly down-regulated. Current work is in progress to determine how and when this occurs.

The findings reported here raise a number of important questions with regard to infection with *T. annulata* and *T. parva*. If these *in vitro* results reflect the situation *in vivo*, there are several possible consequences relating to the pathogenesis of the diseases. The cell types infected by *T. annulata* already express class II antigens and maintain them despite infection. Therefore there is no a priori reason why presentation of *T. annulata* antigens to the immune system should not occur immediately. However, the cell types infected by *T. parva* do not generally express class II molecules. These would have to be switched on before infected cells could be recognized by T helper (Th) cells. Thus the initiation of an immunological response to *T. parva* may be slower than that with *T. annulata*. This may help to explain why there is higher morbidity and mortality with *T. parva* than with *T. annulata*.

It has been shown that an important effector mechanism during both *T. parva* (Morrison et al., 1988) and *T. annulata* (Innes et al., 1989a) infection involves class I-restricted T cytotoxic (Tc) cells. Moreover, cell lines infected with *T. annulata* or *T. parva* both express MHC class I antigens to a similar degree (Spooner & Brown, 1980). Thus the differences between the two diseases would not appear to be explained by differences in CTL function.

The fact that *T. annulata* infects antigen-presenting cells (APC) whereas *T. parva* infects non-APC may lead to differences in the functioning of the immune system and may affect not only the response to the diseases themselves but also the response to intercurrent infections. Infection of APC by *T. annulata* may suppress their ability to present third-party antigens. The initial characterization of the mAb IL-A24 suggests that the molecule that it recognizes is involved in antigen presentation (Ellis et al., 1987) and more recently we have demonstrated the antigen-presenting cell function in cattle is associated with IL-A24-positive, MHC class II-positive adherent cells (Glass & Spooner, 1989). The down-regulation of this antigen may adversely affect APC function. Conversely *T. parva* infection of T cells may suppress the ability of Th cells to help or Tc cells to kill. However, an alloreactive cytotoxic T-cell line did maintain cytotoxic function for a limited period after infection with *T. parva* (Baldwin & Teale, 1987). We are currently investigating these possibilities.

There are further differences between the two parasites. With *T. annulata*, cell lines generated both *in vivo* (Pipano, 1981) and *in vitro* (Ouhelli et al., 1989) can be used to immunize genetically unrelated cattle. By contrast, with *T. parva* only high doses of allogeneic infected cells will immunize cattle (Brown, 1981). We have shown that at low cell doses cattle are only immunized against *T. parva* if the recipient animal and the immunizing cell line have the same major histocompatibility complex (MHC) class I BoLA type (Teale, 1983; Dolan et al., 1984). This histocompatibility barrier to immunization does not
occur with *T. annulata*, even with as few as 100 allogeneic cells (Innes et al., 1989b). It is not clear how the different cell preferences of the two parasites could affect the ease of immunization. If schizonts (the intracellular stage of the parasite) have the same cellular preferences in vivo as we have shown for sporozoites in vitro this might help to explain why it is easier to infect and immunize animals with *T. annulata* than with *T. parva*.

*T. annulata* and *T. parva* thus provide a unique model for dissecting the bovine immune system that may have wider relevance to immunological questions in other species.

**ACKNOWLEDGMENTS**

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