Metastasis of *Theileria annulata* macroschizont-infected cells in *scid* mice is mediated by matrix metalloproteinases

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SUMMARY

Theileria annulata (Ta)-infected leucocytes are able to disseminate in *scid* mice. The dose of virulent parasites of the Ta-Ode line required to achieve quantifiable dissemination was found to be 2×10^6 cells given i.p. Dissemination was higher on day 11 post-inoculation than on day 18. The attenuated Ta-Ode cells were found to disseminate very poorly compared to their virulent progenitors, which correlates with a marked reduction in matrix metalloproteinase (MMP) expression. A daily i.p. injection of mice with BB94, a synthetic inhibitor of MMPs, almost completely ablated dissemination compared to controls. This provides strong evidence that metastasis of *Theileria annulata* macroschizont-infected host cells is mediated by host MMPs induced by the parasite. This has important implications for explaining a number of pathological features of tropical theileriosis in cattle.

Key words: *Theiliera annulata*, macroschizont-infected cells, *scid* mice, metastasis, matrix metalloproteinase, BB94, attenuation.

INTRODUCTION

An important feature of the lymphoproliferative disease of cattle, tropical theileriosis, is the establishment of foci of replicating macroschizont-infected cells in the organs of infected cattle (Srivastava & Sharma, 1981; Eisler, 1989; Forsyth, 1997). These foci resemble multicentric tumours (Irvin & Morrison, 1987). Metastasizing tumour cells produce proteolytic enzymes which aid their migration across the basement membranes of organ systems, allowing the formation of secondary tumours. One class of proteinases known to play an important role in metastasis are the matrix metalloproteinases (MMPs) (Brown, 1993; Brown *et al.* 1993*a*,*b*; Davies *et al.* 1993; Stetler-Stevenson, Aznavoorian & Liotta, 1993*a*; Stetler-Stevenson, Liotta & Kleiner, 1993*b*; Bernhard, Gruber & Muschel, 1994; Chirivi *et al.* 1994; Iwata *et al.* 1996; Sier *et al.* 1996). Baylis *et al.* (1992) demonstrated that the infection of bovine leucocytes with *T. annulata* resulted in the *de novo* expression of 8 novel proteinase activities. The major secreted activity was shown to be bovine MMP9 by cloning and sequence analysis (Baylis, Megson & Hall, 1995). All 8 activities were inhibited by tissue inhibitor of metalloproteinase-1 (TIMP-1) and by 4 synthetic hydroxamic acid-based MMP inhibitors, providing strong evidence that they are all MMPs (Adamson & Hall, 1996). *T. annulata*-infected leucocytes can cross a reconstituted basement membrane (Matrigel™) in an *in vitro* assay of metastasis and this transmigration was markedly reduced by up to 90% when MMP inhibitors were present (Adamson & Hall, 1996). We have, therefore, postulated that these induced host MMP enzymes are necessary for the *in vivo* dissemination of macroschizont-infected leucocytes, allowing the establishment of tumour-like foci (Adamson & Hall, 1997).

The virulence of *T. annulata*-infected cells can be attenuated by prolonged *in vitro* cultivation and this process is used to generate effective live vaccines (Pipano, 1981; Hall, 1988; Tait & Hall, 1990; Singh, 1990). Attenuation results in the loss of MMP activity in all vaccine lines examined and this may partly explain the loss of virulence (Baylis *et al.* 1992; Somerville, 1997). In support of this idea we observed that an attenuated vaccine cell line, *Theiliera annulata*-Ode (Ta-Ode), had a reduced ability to metastasize *in vitro* when compared with its non-attenuated progenitor (Adamson & Hall, 1996).

There is no rodent model of theileriosis although several groups have attempted to establish *Theileria* infections in immunodeficient mice (Irvin *et al.* 1977; Fell & Preston, 1992, 1993). *T. annulata*-infected cells can be made to disseminate throughout the body of *scid* mice following intraperitoneal inoculation (Fell & Preston, 1993). In this study we

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used the *scid* mouse system to examine the role of MMPs in the *in vivo* dissemination of *T. annulata*-infected leucocytes. We show that the attenuated Ta-Ode line, which has diminished MMP expression, disseminates very poorly compared with its virulent counterpart. Also, dissemination is dependent on the expression of MMPs since it can be abolished by BB94 (Batimastat), a hydroxamic acid-based inhibitor of the enzymes (Brown, 1995).

MATERIALS AND METHODS

Cell lines and cell culture

The non-attenuated (p58) and attenuated (p158) Ta-Ode vaccine cells (Singh, 1990) were cultured *in vitro* using the method of Brown (1987). Macroschizont-infected cells were cultured in RPMI 1640 medium supplemented with 16 % foetal bovine serum (Gibco BRL), 25 mM Hepes, 4 mM L-glutamine, 100 i.u./ml penicillin, 100 μg/ml streptomycin, 0·75 μg/ml Fungizone, 10 μM 2-mercaptoethanol (BDH) at 37 °C with 5 % CO_2 in air.

Infection studies using scid *mice*

C.B.–17 *scid* mice were obtained from Harlan, UK Ltd. The mice were housed in flow cabinets (Kytötä, Muurame, Finland) and were fed on autoclaved food and water. All animals were female aged between 3 and 4 months on the first day of the experiment. *T. annulata*-infected cells were cultured in antibiotic-free medium for 2 weeks prior to inoculation into mice. *T. annulata*-infected cells had their viability established by Trypan blue (Sigma) staining. The cells were pelleted by centrifugation at 1100 ***g*** and resuspended in PBS at an appropriate concentration of live cells. Groups of 10 mice were inoculated by intraperitoneal injection with either 2×10^6 or 2×10^7 Ta-Ode infected cells. Where appropriate, mice were inoculated i.p. daily with 30 mg/kg of BB94 (kindly donated by British BioTechnology). The BB94 was made as a 6 mg/ml suspension by sonication in PBS with 0·01 % Tween 20 (Sigma) (Chirivi *et al*. 1994).

Preparation of tissue smears

Mice were killed on either day 11 or day 18 and the tumours and organs of interest (lung, liver, spleen, kidney and mesentery) were promptly removed and rinsed in PBS. Impression smears were prepared by cutting each organ in half and dragging the cut surface over a microscope slide and then Giemsa stained. Counts of total macroschizonts (i.e. both intracellular and extracellular) were made by scanning 100 random fields of view. The results are expressed as numbers of macroschizonts per field.

RESULTS

Titration of Ta-Ode-infected cells for optimum dissemination

It was necessary to establish a suitable inoculum of infected cells that would disseminate from the site of inoculation to the major organ systems of the *scid* mice. Initially, mice were infected with 2×10^6 and 2×10^7 Ta-Ode-infected virulent (p58) cells. Ten mice were infected with each dose. The higher dose was lethal resulting in death of 9 out of the 10 animals on day 11. Five of the animals inoculated with 2×10^6 were sacrificed on day 11 and the other 5 were sacrificed on day 18 of the experiment. The degree of dissemination using 2×10^6 Ode-infected cells is shown in Fig. 1. At day 11 all the organs sampled contained either free macroschizonts or macroschizont-infected cells. The term 'tumour' refers to a bloody mass that appeared on the wall of the abdomen around the site of inoculation. The dissemination in the animals killed on day 18 was lower than on day 11. Therefore, all further experiments were carried out for 11 days only.

Virulent Ta-Ode cells disseminate much more than attenuated Ta-Ode cells

We have assessed the ability of the virulent (p58) and attenuated (p158) Ta-Ode cells to metastasize into the organs of *scid* mice. Ten mice were inoculated with 2×10^6 virulent Ta-Ode-infected cells and another 10 were inoculated with 2×10^6 attenuated Ta-Ode-infected cells. After 11 days both groups of mice were killed and impression smears made of their organs. The results are presented graphically in Fig. 2. Schizonts and schizont-infected cells were seen in all the organs of mice infected with virulent cells. In contrast, animals inoculated with attenuated cells showed no schizont-infected cells in the organs of the mice, except for the lungs.

BB94 ablates the ability of macroschizont-infected cells to invade organs

We examined the effect of the MMP inhibitor, BB94, on the dissemination of *T. annulata*-infected cells in *scid* mice. Thirty animals were inoculated with 2×10^6 virulent Ta-Ode-infected cells and subsequently divided into 3 groups. Ten of the animals were left untreated, 10 were injected with PBS and 0·01 % Tween 20 daily and 10 were injected daily with BB94 suspended in PBS/0·01 % Tween 20. After 11 days all the animals were killed and impression smears made of their organs. The results are graphically represented in Fig. 3. Untreated controls and the diluent-inoculated controls had significant numbers of macroschizont-infected cells disseminated throughout their organs, whilst the

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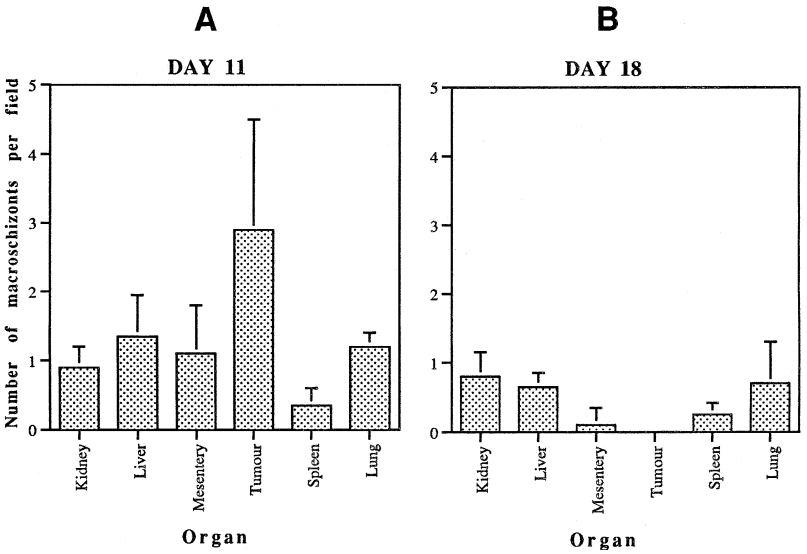

Fig. 1. The effect of different end-point times on the dissemination of virulent (p58) Ta-Ode macroschizonts in *scid* mice. A total of 2×10^6 macroschizont-infected leucocytes was inoculated i.p. into female *scid* mice. Five of the mice were killed on day 11 (A) and 5 on day 18 (B). Impression smears of the tissues listed were prepared and stained with Giemsa. Macroschizonts were counted in 100 random fields for each organ from each mouse and the data are presented as the mean number of schizonts per field per tissue $\pm$ the standard deviation. The term 'tumour' is used to describe a bloody mass that appeared on the abdominal wall at the site of the inoculation.

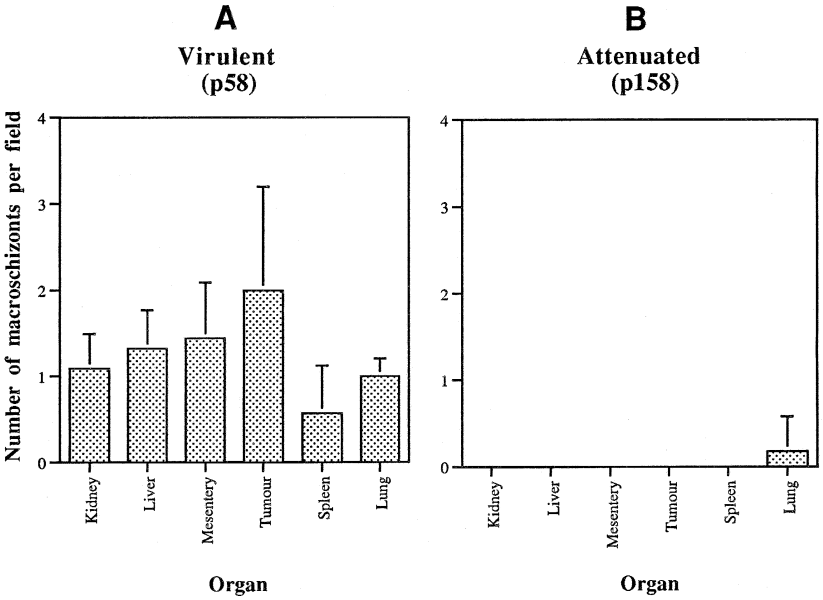

Fig. 2. Comparison of the dissemination of the virulent (non-attenuated, p58) (A) and the attenuated (p158) (B) Ta-Ode cells. Each group of 10 *scid* mice was inoculated i.p with 2×10^6 macroschizont-infected leucocytes. Mice were killed on day 11 and analysed as stated in the legend to Fig. 1.

BB94-treated group had very few parasites and these were restricted to the liver and lung. These data establish that MMPs are necessary to mediate metastasis of *Theiliera*-infected cells.

DISCUSSION

We have previously suggested that the host MMPs expressed by *T. annulata*-infected leucocytes are necessary for their metastatic behaviour (Adamson & Hall, 1996, 1997). This idea is based on (i) the extensive evidence that this class of enzyme has been greatly implicated as a causal factor in the metastasis exhibited by a range of tumour cells (Stetler-Stevenson *et al.* 1993*b*; Brown, 1995) and (ii) our recent experiments demonstrating that a range of MMP inhibitors block the transmigration of macroschizont-infected cells through Matrigel™ (Adamson & Hall, 1996). In the present study we sought to extend our findings to an *in vivo* model. We chose *scid* mice since it had previously been shown that *T. annulata* disseminates to the organs of this strain

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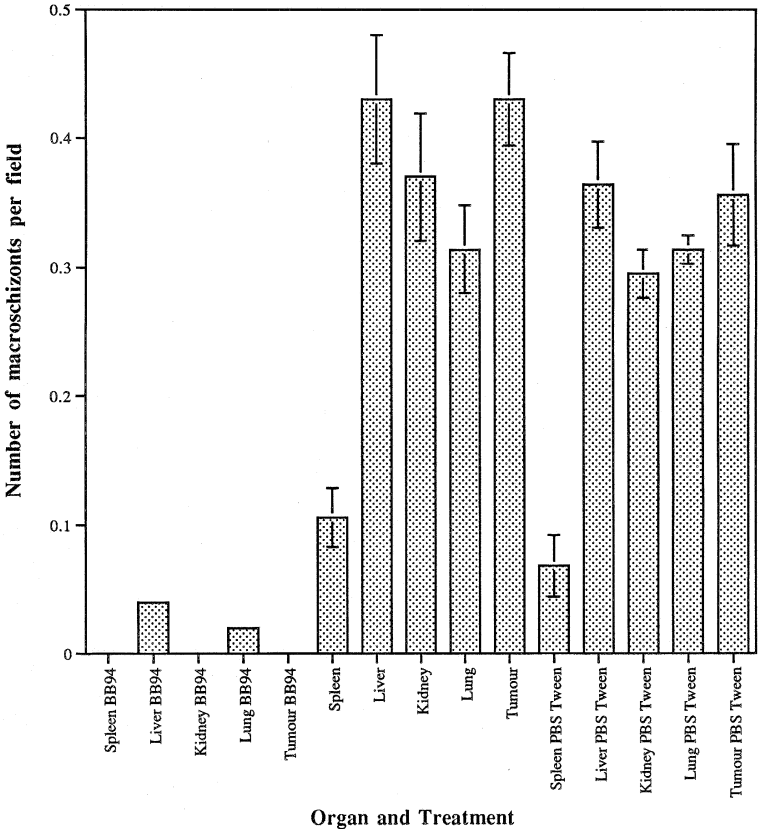

Fig. 3. Effect of BB94 on the dissemination of virulent (p58) Ta-Ode macroschizonts in *scid* mice. Three groups of 10 mice were inoculated i.p. with 2×10^6 macroschizont-infected leucocytes. One group was given a daily i.p injection of BB94 in PBS/Tween 20, 1 group was given the PBS/Tween 20 only and 1 group was untreated. Mice were killed on day 11 and analysed as stated in the legend to Fig. 1.

(Fell & Preston, 1993). The parasite stock chosen was the Ta-Ode vaccine line since the virulent early form (p58) produced abundant MMP activity and was the most aggressive in the Matrigel™ assay (Adamson & Hall, 1996). In addition, a comparison could be made with the attenuated form (p158) which has much diminished MMP levels and a correspondingly reduced ability to cross Matrigel™ (Adamson & Hall, 1996). Initially it was necessary to optimize both the size of the Ta-Ode inoculum and the time-scale of the assay to ascertain the best conditions for achieving dissemination from the site of inoculation to the organs of the *scid* mice. Our results indicate that a dose of 2×10^6 non-attenuated Ta-Ode-infected cells is suitable. This dose of cells differs from that previously reported in the literature by Fell & Preston (1993), who reported that a dose of 2×10^7 Ta-Hissar cells is suitable for this purpose. We tried this higher dose of cells with the virulent (p58) Ta-Ode vaccine cell line but this turned out to be lethal on day 11 of the experiment. This observation probably reflects differences in the natural virulence of these 2 *T. annulata* isolates, with the Ta-Ode-infected cell line being more virulent than the Ta-Hissar line. This means that when experiments are performed with different isolates of *T. annulata*, the number of infected cells will need to be titrated each time. The number of infected cells in the organs of *scid* mice drops between the 11 and 18 day time-points. A similar observation was made by Fell & Preston (1993) and is probably due to the innate immunity (macrophages and NK cells) that *scid* mice retain. For these reasons we chose the day 11 as our end-point for the remaining experiments.

We compared the ability of the attenuated Ta-Ode (p158) vaccine cell line to metastasize in the organs of *scid* mice with their virulent (p58) progenitor. The results show that the virulent cells invade all the organs of *scid* mice sampled whereas the attenuated cells do not disseminate. This mirrors the previous *in vitro* data showing that the virulent cells go through Matrigel more readily than the attenuated cells (Adamson & Hall, 1996). Both these results are probably because the attenuated cells do not express much MMP activity (Bayliss *et al*. 1992) and are thus unable to degrade the basement membranes of organs. Invasion of tissues is a multifactorial process involving cell–matrix adhesion, proteolytic degradation and migration across the basement membrane. Thus differences in other molecules (in addition to MMPs) involved in invasion could be responsible, at least in part, for the altered invasiveness of the non-attenuated and attenuated lines.

To prove that MMPs have a crucial role in the *in vivo* dissemination of Ta-Ode cells we demonstrated that the synthetic MMP inhibitor BB94, shown to

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inhibit the metastatic spread of both human tumour xenografts in nude mice and murine melanomas in C57BL/6J mice (Brown, 1995), severely reduced the ability of these cells to infiltrate the organs of *scid* mice. The result obtained mirrored our previous data where BB94 was shown to inhibit the migration of Ta-Ode-infected cells in an *in vitro* assay of metastasis (Adamson & Hall, 1996). Based on these data we believe that the induction of host MMPs by infection with *T. annulata* macroschizonts confers upon them a metastatic phenotype. Thus, we suggest that host MMPs are virulence determinants and their inappropriate expression causes pathology which as well as including the ability to disseminate may also be the basis for the ulceration of the abomasum commonly found in tropical theileriosis. Studies to prove that MMPs have a role in the true *in vivo* situation in cattle are now required and will form the next phase in this line of research.

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