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Expression of T-Cell-Associated Serine Proteinase 1 during Murine *Leishmania major* Infection Correlates with Susceptibility to Disease

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The expression of T-cell-associated serine proteinase 1 (MTSP-1) in vivo during *Leishmania major* infection was analyzed in genetically resistant C57BL/6 mice and in genetically susceptible BALB/c mice. Using a monoclonal antibody as well as an RNA probe specific for MTSP-1 to stain tissue sections, we found T cells expressing MTSP-1 in skin lesions and spleens of mice of both strains. In skin lesions, MTSP-1-positive T cells could be detected as early as 3 days after infection. Most importantly, the frequency of T cells expressing MTSP-1 was significantly higher in susceptible BALB/c mice than in resistant C57BL/6 mice. These findings suggest that MTSP-1 is associated with disease-promoting T cells and that it may be an effector molecule involved in the pathogenesis of cutaneous leishmaniasis.

Protozoan parasites of the genus *Leishmania* cause a spectrum of human diseases ranging from self-healing cutaneous ulcers to fatal visceral dissemination, depending on both the host's immune response and the species of parasite. The distinct disease patterns can be reproduced experimentally in mice. After infection with *Leishmania major*, the cause of cutaneous leishmaniasis, mice of genetically resistant inbred strains (e.g., C57BL/6) can contain the infection with cutaneous lesions healing spontaneously, whereas genetically susceptible mice (e.g., BALB/c) develop progressive cutaneous ulceration and visceralization and eventually die. It has been shown that both resistance and susceptibility to disease are elicited by the $CD4^+$ T-cell subpopulation (10, 16).

Previous studies have shown that T effector cells synthesize a variety of soluble mediators, such as lymphokines and enzymes, which are secreted upon antigenic stimulation (25, 26). Among other proteins, cytoplasmic granules of activated T cells contain a serine proteinase (20), termed synonymously murine T-cell-associated serine proteinase 1 (MTSP-1; 22), granzyme A (12), serine esterase 1 (27), and Hanukah factor (5), which is produced in vitro by all $CD8^+$ and a fraction of $CD4^+$ T cells (4). The cDNA for this enzyme has been cloned (5). In vivo, MTSP-1 has been demonstrated in T cells previously sensitized during viral infections (8, 18), allograft rejection (17), or autoimmune diseases (19).

Preliminary studies on the possible function of MTSP-1 indicated that the enzyme might be involved in T-cell-mediated processes, such as the control of virus replication (8, 18), extravasation (24), and inflammation (1). In the present study on the potential involvement of MTSP-1 in the pathology of murine cutaneous leishmaniasis, we analyzed the expression of MTSP-1 in infected mice by both immunocytochemistry and in situ hybridization with RNA probes.

The cloned virulent parasite isolate used for this study was confirmed to be *L. major* by isoenzyme analysis (D. Evans, London School of Hygiene and Tropical Medicine, London, England) and was maintained by passage in BALB/c mice. Promastigotes were grown in vitro in blood agar cultures. Stationary-phase promastigotes (2×10^6) were injected in a volume of 50 μl intradermally on the dorsum of the mouse close to the base of the tail. Female mice of the inbred strains BALB/c and C57BL/6 were purchased from Charles River Wiga Breeding Laboratories (Sulzfeld, Federal Republic of Germany). Lesion scores were determined according to the following system: 0 = no lesion; 1 = small swelling (up to 5 mm in average diameter); 2 = large swelling (more than 5 mm in average diameter) or open lesion of less than 5 mm in average diameter; 3 = open lesion of more than 5 mm in average diameter.

For immunohistological analysis of MTSP-1 expression, frozen sections (4 μm) of organs from infected mice were stained with a rat monoclonal antibody recognizing MTSP-1 (8). Monoclonal rat anti-mouse CD4 and CD8 antibodies were derived from the hybridomas H129.19 (21) and 53-6.7 (9), respectively. A three-step streptavidin-biotin-peroxidase (ABC) method was used as previously described (8). The preparation of ^{35}S-labeled RNA probes of the MTSP-1 gene (Hanukah factor gene [5]) and the in situ hybridization of cryostat sections were done as previously described in detail (17, 18). Multiple sections from each tissue were hybridized with either an antisense RNA probe or a sense RNA probe, the latter as a nonhybridizing control which uniformly gave negative results. Spleens from mice infected with lymphocytic choriomeningitis virus, known to be positive for expression of MTSP-1 (8, 18), and tissues from uninfected mice were included in each experiment as a positive and negative control, respectively.

C57BL/6 and BALB/c mice were infected intradermally with *L. major*, and at different time intervals thereafter, lesional skin, draining lymph nodes, and spleens were removed and analyzed by immunocytochemistry. Figure 1 illustrates the course of cutaneous lesion development in the two strains of mice. In the initial series of experiments, we analyzed the distribution of $CD4^+$ and $CD8^+$ T cells. At any time of infection tested, the distribution pattern as well as

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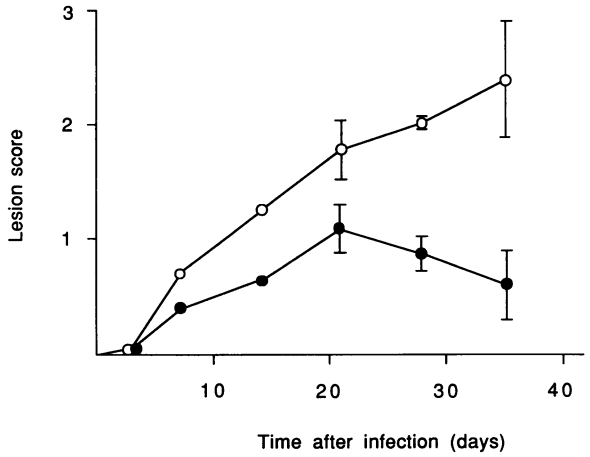

FIG. 1. Comparison of cutaneous infection in susceptible and resistant mice. BALB/c (○) and C57BL/6 (●) mice were infected intradermally with 2×10^6 *L. major* promastigotes. Arithmetic means of the cutaneous lesion scores and standard errors are given for certain time points.

the ratio of $CD4^+$ to $CD8^+$ T cells in sections of spleens and lymph nodes were comparable to those of uninfected control mice (data not shown). Analysis of the lesional skin, however, revealed that on day 3 after infection, T cells infiltrating the lower dermal compartment were exclusively of the $CD4^+$ phenotype. This was found for mice of both strains, the number of $CD4^+$ cells being significantly higher in BALB/c mice (Fig. 2A) than in C57BL/6 mice (Fig. 2B). No $CD8^+$ T cells were observed at this time point (as shown for BALB/c in Fig. 2C). At later stages of disease, the number of $CD8^+$ cells in the lesions increased, particularly in C57BL/6 mice, but remained consistently lower than that of $CD4^+$ cells (Table 1).

In skin lesions of susceptible BALB/c mice, numerous T cells expressing MTSP-1 could be detected by immunostaining as early as 3 days after infection (Fig. 2D), at a time when all the infiltrating lymphocytes were shown to be of the $CD4^+$ phenotype (Table 1; Fig. 2A and C). MTSP-1 expression was strongest in the early phase of infection, before the appearance of open lesions (at approximately 3 weeks after infection). In resistant C57BL/6 mice, on the other hand, the

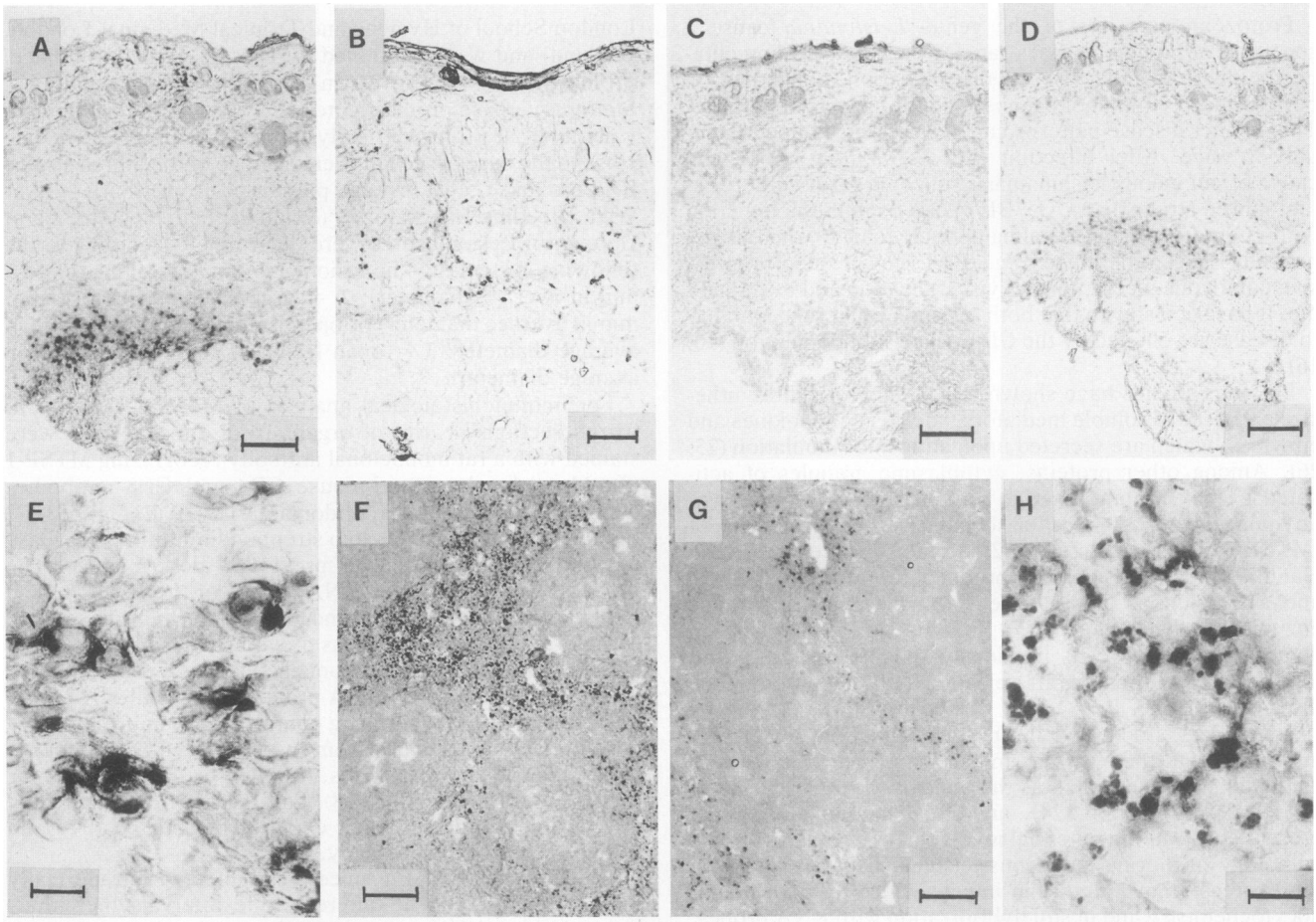

FIG. 2. Immunohistochemical analysis of tissue sections from mice infected with *L. major* (ABC method, hematoxylin counterstaining). $CD4^+$ cells are abundant in lesional skin of BALB/c mice (A) and are much less frequent in C57BL/6 mice (B) on day 3 after infection. As demonstrated in panel C for BALB/c mice, no $CD8^+$ cells can be detected in this organ at the same time point. High levels of MTSP-1 expression are found in the cutaneous lesions (D and E; day 3) and the spleens (F and H; day 14) of BALB/c mice. MTSP-1-positive cells can also be detected in the spleens of C57BL/6 mice (G; day 14), but their percentage is markedly lower. Scale bars: A, C, D, F, and G, 90 μm; B, 70 μm; E, 10 μm; H, 20 μm.

TABLE 1. Expression of T-cell surface markers CD4 and CD8 in cutaneous lesions of *L. major*-infected mice[a]

Mouse strain	Antigen detected at the following day after infection[b]							
	3		7		14		21	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
C57BL/6	+	−	++	+	+++	++	++	+
BALB/c	++	−	+++	+	+++	+	++	+

[a] Immunohistochemistry was performed as described previously (8). The results represent means of two experiments with two mice for each time point, respectively.

[b] −, negative (comparable to Fig. 1C); +, less than 5% positive cells in scattered distribution (comparable to Fig. 1B); ++, 5% to 20% positive cells (comparable to Fig. 1A); +++, more than 20% positive cells.

number of T cells expressing MTSP-1 remained markedly lower (Table 2).

To investigate whether the high levels of MTSP-1 protein expression in *L. major*-infected BALB/c mice were due to transcriptional or translational regulation, we performed in situ hybridization experiments using RNA probes of the MTSP-1 gene. In lesional skin, a high number of cells displayed strong hybridization signals as demonstrated by the dense cell-associated silver grain accumulation (day 14 after infection; Fig. 3A and B). In contrast, MTSP-1 mRNA expression could not be detected in infected C57BL/6 mice (Fig. 3C) despite the abundance of infiltrating lymphocytes.

T cells expressing MTSP-1 were also detected by immunostaining of spleen sections from mice infected with *L. major* (Table 2). As for skin lesions, the number of splenic T cells expressing MTSP-1 was significantly higher in susceptible BALB/c mice than in resistant C57BL/6 mice. This difference was most pronounced on day 14 after infection (Fig. 2F and G). In spleens (Fig. 2H) and in cutaneous lesions (Fig. 2E), MTSP-1 was confined to intracellular granular structures of T cells. Only few MTSP-1$^+$ T cells were detected in lymph nodes draining the lesions (data not shown). Cells expressing MTSP-1 were absent in sections of the spleen and skin from uninfected mice (Table 2).

The present study suggests the involvement of the proteolytic enzyme MTSP-1 in the pathogenesis of *L. major* infection in mice. In the course of cutaneous disease, the frequency of T cells expressing MTSP-1 was much higher in spleens and skin lesions of susceptible BALB/c mice than in those of resistant C57BL/6 mice. This significant difference

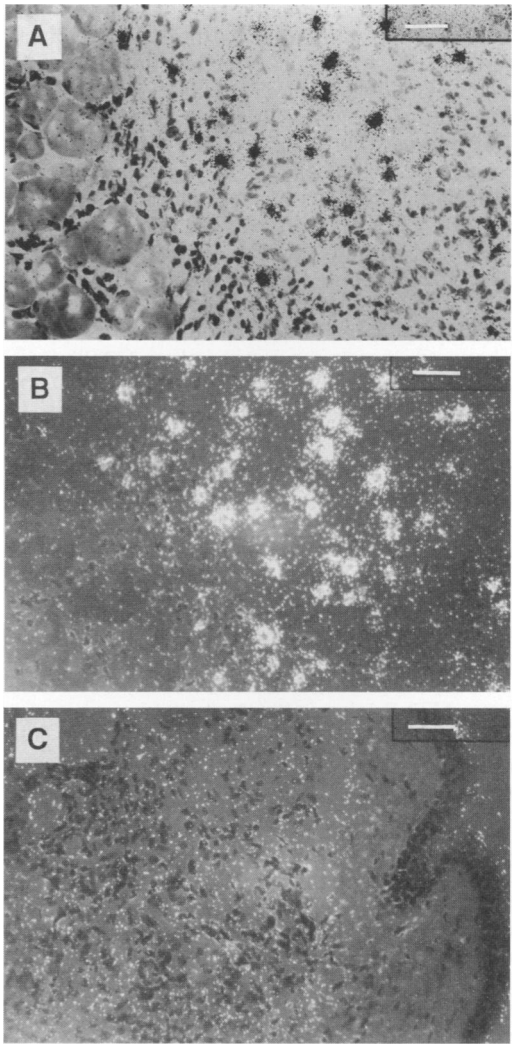

FIG. 3. In situ hybridization for detection of MTSP-1 mRNA in skin lesions at 2 weeks after infection with *L. major*. The bright-field photomicrograph shows a skin section of a BALB/c mouse (A) with a high percentage of MTSP-1 gene-expressing cells which are easily seen by epipolarization illumination (B). No MTSP-1 mRNA-positive cells were detectable in C57BL/6 mice (C; combination of bright-field and epipolarization illumination). Scale bars = 25 μm.

TABLE 2. Expression of MTSP-1 in *L. major*-infected mice[a]

Source	Expression at the following day after infection[b]:					
	0	3	7	14	21	28
Skin/cutaneous lesion						
C57BL/6	−	−	+	+	+	+
BALB/c	−	++	++	++	+	+
Spleen						
C57BL/6	−	+	+	+	+	+
BALB/c	−	+	+	+ ⌐ ++	++	+

[a] Immunohistochemistry was performed as described previously (8). The results represent means of two experiments with two mice for each time point, respectively.

[b] −, negative (comparable to Fig. 1C for cutaneous lesion); +, less than 5% positive cells in scattered distribution (comparable to Fig. 1G for spleen); ++, 5 to 20% positive cells (comparable to Fig. 1D for cutaneous lesion); +++, more than 20% positive cells (comparable to Fig. 1F for spleen).

was demonstrated on the translational level by using an antibody specific for the MTSP-1 protein as well as on the transcriptional level by using radiolabeled probes that detect MTSP-1 mRNA. Staining of sequential sections and determination of the enzyme activity of purified lymphocytes in vitro confirmed that MTSP-1 expression in infected mice was associated with T cells (data not shown). Thus, it is tempting to speculate that in murine *L. major* infection, MTSP-1 is associated with disease-promoting rather than resistance-promoting T cells. In accordance with this assumption is the interesting observation that MTSP-1 expression was also enhanced in an unusually severe skin lesion of an individual C57BL/6 mouse with no sign of healing at day 35 after infection (data not shown). We have previously demonstrated that C57BL/6 mice presenting with such an unusual course of disease have an increased frequency of disease-promoting T cells (14). This finding emphasizes that

MTSP-1 expression in infected C57BL/6 mice is not generally decreased but is related to the severity of cutaneous leishmaniasis. During the immune response to another infectious agent, lymphocytic choriomeningitis virus, T cells from C57BL/6 mice also express high levels of MTSP-1 (8, 18).

It is of interest that we consistently detected only a few MTSP-1-expressing T cells in the lymph nodes draining the lesions of BALB/c mice. At present, the significance of this finding is unclear. It is possible that this small population of MTSP-1$^+$ cells contains a particularly high proportion of *L. major*-specific lymphocytes and/or that MTSP-1 is expressed only after migration of *L. major*-reactive T cells from the lymph nodes to the cutaneous lesion.

Previous studies have shown that MTSP-1 is mainly associated with cytolytic CD8$^+$ T cells in vitro (12, 20, 22, 27) and in vivo (8) and that only a fraction of murine (4) or human (11) CD4$^+$ T cells express MTSP-1 in vitro. Our own findings in mice (3) and a recent report by Cooper et al. (2) on T-cell subsets in human leprosy lesions strongly suggested the existence of CD4$^+$ T cells expressing MTSP-1 or its human analog in vivo. In the present study, high numbers of T cells expressing MTSP-1 were observed in *L. major*-infected skin sections that did not display CD8$^+$ cells. Moreover, the location of these MTSP-1-containing cells was identical to that of CD4$^+$ cells. These findings underscore the capacity of both T-cell subsets, CD8$^+$ and CD4$^+$, to express MTSP-1 in vivo.

Our immunohistological examinations of *L. major*-infected skin consistently demonstrated the presence of T cells not only in lesions of C57BL/6 mice but also in those of BALB/c mice. On the other hand, a previous report (13) showed that the T-cell influx into the infected dermis of BALB/c mice is minimal. This discrepancy may be explained by the use of a different parasite strain, *L. mexicana amazonensis*, in the latter study because, even in self-healing C57BL/6 mice, cutaneous *L. mexicana amazonensis* infection is associated with a overall delayed T-cell response in the skin lesion (13). In accordance with McElrath et al. (13), we found that the ratio of CD4$^+$ to CD8$^+$ T cells in spleen and lymph node sections did not change during infection for either strain of mice, although the absolute number of T cells increased in amounts proportional to the overall enlargement of these organs. However, on the basis of tissue sections, it is difficult to estimate the total number of T cells per organ, and this may be the reason why others (7), after labeling of isolated cell suspensions, have found different population dynamics during infection.

Analysis of the time course of MTSP-1 expression revealed that T cells containing the enzyme were detectable as early as 3 days after infection. Together with the fact that the number of lymphocytes expressing MTSP-1 decreased at later stages of chronic disease, this implies that MTSP-1 is involved in early events leading to the development of cutaneous lesions. Our recent finding that MTSP-1 is able to cleave structural molecules of the extracellular matrix, such as fibronectin (23), collagen type IV (24a), and sulfated proteoglycans (24), suggests that this proteolytic enzyme directly contributes to tissue destruction and development of ulcerative lesions. Proteolytic degradation of extracellular matrix proteins may furthermore facilitate recruitment of other cell populations required for subsequent completion of lesion formation. These effects may even be enhanced by MTSP-1-mediated activation of the plasminogen activator/plasmin system (1), a potent system of extracellular proteolysis. Future studies will be addressed to the possible involvement of MTSP-1 in pathophysiological processes, including ulcerative inflammation.

The present data indicate that disease-promoting CD4$^+$ cells may differ from resistance-promoting CD4$^+$ cells not only in the pattern of lymphokine production (6, 15) but also in the expression of the proteolytic enzyme MTSP-1. It will now be of interest to determine the frequency and antigen specificity of CD4$^+$ MTSP-1$^+$ T cells from *L. major*-infected mice and to study their functional capacity including the pattern of lymphokine production. Current work is focused on this aspect.

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