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Cutaneous leishmaniasis: co-ordinate expression of granzyme A and lymphokines by CD4$^+$ T cells from susceptible mice

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SUMMARY

We have recently demonstrated that the frequency of T cells expressing granzyme A is significantly higher in skin lesions and spleens of susceptible BALB/c mice compared with resistant C57BL/6 mice infected with *Leishmania major*, a cause of human cutaneous leishmaniasis. In the present study, we have performed *in vitro* studies to characterize the subpopulation, the antigen responsiveness and the lymphokine production pattern of granzyme A-expressing T cells in *L. major*-infected mice. Using a limiting dilution system for functional analysis of selected T cells at the clonal level, we could show that granzyme A activity in infected BALB/c mice can be assigned to *L. major*-reactive CD4$^+$ T cells secreting interleukin-2 (IL-2) and IL-4. Granzyme A production was most pronounced in the early phase of infection. On the other hand, granzyme A expression could not be detected in C57BL/6-derived T cells responding to *L. major*. The data support the suggestion that granzyme A is produced by *L. major*-responsive CD4$^+$ T cells facilitating lesion formation and the dissemination of infection.

INTRODUCTION

The range of disease manifestations observed in humans infected with protozoan parasites of the genus *Leishmania* can be imitated experimentally in mice. After infection with *L. major*, mice of resistant inbred strains, such as C57BL/6, develop self-healing cutaneous ulcers. On the other hand, the same treatment has a fatal impact for susceptible BALB/c mice because the parasites disseminate from the infected skin to the viscera. These genetically determined differences in the outcome of infection have been attributed to the lymphokine secretion pattern of *L. major*-reactive CD4$^+$ T cells.[1,2] The presence of interleukin-12 (IL-12) favours the development of interferon-γ (IFN-γ)-producing T cells and healing of skin lesions, whereas the production of IL-4 and IL-10 correlates with suceptibility to disease.[3–8]

We have recently provided evidence that disease-promoting CD4$^+$ cells may differ from resistance-promoting CD4$^+$ cells not only in the pattern of lymphokine production but also in the expression of a serine proteinase.[9] This enzyme, designated granzyme A[10] or synonymously murine T-cell-associated serine proteinase (MTSP-1),[11] serine esterase 1[12] or Hanukah factor,[13] is stored in cytoplasmic granules of activated T cells,[10,14,15] and is released by receptor-triggered exocytosis.[16] Granzyme A expression has been demonstrated in T cells sensitized during viral infections,[17,18] allograft rejection[19] or autoimmune disease.[20] It is mainly associated with CD8$^+$ T cells[10–12,15,17] and only a fraction of CD4$^+$ cells has been found to express the enzyme *in vitro*.[21] However, it is not known whether the differential granzyme A activity of CD4$^+$ cells correlates with the ability to release lymphokines.

In murine leishmaniasis, we showed that the frequency of T cells expressing granzyme A was significantly higher in susceptible BALB/c mice than in resistant C57BL/6 mice.[9] Furthermore, granzyme A expression in BALB/c skin lesions was associated with CD4$^+$ T cells. We have now tested whether the capacity of T cells from *L. major*-infected mice to express granzyme A correlates with their lymphokine activitiy. Using a limiting dilution (LD) system for clonal analysis of short-term cultured lymphocytes, it was possible to quantify parasite-reactive T cells containing granzyme A and to determine whether enzyme activity and lymphokine activity were elicited by the same or by separate T-cell populations.

MATERIALS AND METHODS

Parasites and infection of mice

The origin and propagation of the *L. major* isolate have been described elsewhere.[22] The cloned virulent line used for this

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Abbreviations: IFN, interferon; IL, interleukin; LD, limiting dilution; mAb, monoclonal antibodies; SN, supernatant.

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study was confirmed to be *L. major* by isoenzyme analysis and was maintained by passage in BALB/c mice. Promastigotes were grown *in vitro* in blood agar cultures. Stationary-phase promastigotes were washed in phosphate-buffered saline and, for preparation of lysate, were subjected to three cycles of rapid freezing and thawing. For intradermal infection of mice, 2×10^6 organisms were injected in a volume of 50 μl on the dorsum close to the base of the tail.

Female mice of the inbred strains BALB/c and C57BL/6 were 6–12 weeks of age at the onset of experiments. All mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany) and, during experimentation, were maintained under conventional conditions in an isolation facility.

Culture medium and antibodies

Click's RPMI-1640 medium (Gibco Laboratories, Eggenstein, Germany) was supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES buffer, 100 μg/ml penicillin, 100 μg/ml streptomycin (all these supplements purchased from Seromed-Biochrom, Berlin, Germany), 7·5% $NaHCO_3$ and 5×10^{-5} M 2-mercaptoethanol.

Monoclonal antibodies (mAb) directed against CD4, from hybridoma YTS 191.1.2,[23] CD8, from hybridoma YTS 169.4.2,[23] and IL-2, from hybridoma S4B6,[24] were used as ascites fluid. Anti-IL-4 mAb were used as culture supernatant (SN) from hybridoma 11B11.[25] Anti-Thy-1.2 mAb, from hybridoma F7D5, were purchased from Camon (Wiesbaden, Germany). For the preparation of lymphocyte subpopulations by cytotoxic procedures, single-cell suspensions of spleen cells in RPMI-1640/1% FCS at a concentration of 10^7/ml were incubated for 40 min on ice with anti-CD8 (for preparation of $CD4^+$ responder cells), anti-CD4 (for preparation of $CD8^+$ responder cells) or anti-Thy-1.2 (for preparation of T-cell-depleted accessory cells). Thereafter, rabbit complement (Low-Tox M; Cedarlane Laboratories Ltd, Hornby, Canada) at a final dilution of 1/12 was added and the suspensions were incubated for another 20 min at 37°. The cells were washed and the treatment was repeated once more. The contamination with cells of the depleted phenotype was less than 5% ($CD4^+$ population) or 10% ($CD8^+$ population), as judged by staining with fluoresceinated antibodies.

Activation of T-cell subsets in vitro

At various times after infection with *L. major*, spleen cells were prepared from two mice/experimental group. Before conduction of LD analysis, the responder cells were subjected to a cycle of *in vitro* restimulation and rest in bulk cultures, as described previously.[26] Subsequently, limiting numbers of selected $CD4^+$ or $CD8^+$ cells were cultured in round-bottomed microtitre wells with 2×10^5 accessory cells (syngeneic irradiated spleen cells; 2500 rads) in 0·2 ml of culture medium containing 15% of a SN from phytohaemagglutinin-stimulated human tonsil cell cultures as a source of lymphokines. For each T-cell concentration ranging from 10^4 to 62 cells/well, replicates of 24 wells were set up with parasite lysate (equivalent to 2×10^5 organisms) as a source of antigen. After 9 days incubation, microcultures were washed three times to remove exogenous factors derived from the tonsil cell culture SN and were restimulated by adding 2×10^5 T-cell-depleted accessory cells in the presence of antigen in 0·2 ml of culture medium. After 24 hr, 0·16 ml of SN was collected from each well and split into four aliquots for determination of lymphokine activities. The remaining cells were tested for granzyme A activity. Cultures containing accessory cells and antigen but no T cells were used as negative controls. LD cultures were scored positive when the values of enzyme or lymphokine activity exceeded the arithmetic mean of the control wells by more than 3 SD. For quantification of T cells reacting to *L. major*, a second series of LD cultures was set up and restimulated without antigen for each responder cell population. The number of cultures that were positive in the absence of antigen was subtracted from the fraction of cultures responding in the presence of *L. major* antigen for each dose group. Minimal estimates of the precursor frequency for each lymphokine activity were obtained by the minimum χ^2 method from the Poisson distribution relationship between the number of responder cells and the logarithm of the fraction of negative cultures by using a computer program purchased from C. Taswell.[27]

Assay for granzyme A activity

Lysates were prepared by incubating the cells of each LD culture in 50 μl/well of a Tris buffer (0·01 M, pH 7·5) containing 0·1% Triton X-100 for 30 min on ice. Test samples were mixed with 50 μl (0·3 mM) of the chromogenic substrate H-D-Pro-Phe-Arg-NA (Bachem, Heidelberg, Germany) in 0·01 M Tris–HCl, pH 8·5. After an incubation period of 30–60 min at 37°, the absorbance was determined with a microplate reader (Dynatech, Alexandria, VA), using a test wavelength of 405 nm and a reference wavelength of 490 nm. The specificity has been documented previously using a panel of peptide substrates and various enzyme inhibitors.[14] Lysates from the long-term cultured $CD8^+$ T-cell clone 1.3E6, known to express granzyme A activity,[11,28] and from the thymoma line EL-4.F15, which lacks granzyme A activity,[29] were included as positive and negative controls, respectively, in each assay.

Assays for detection of IL-2, IL-4 and IFN-γ activities

For concurrent determination of IL-2 and IL-4 activity, 40-μl aliquots of SN from individual LD cultures were tested for the ability to stimulate proliferation of 3×10^3 cells of the HT-2 T-cell line[30] in a total volume of 0·1 ml in flat-bottomed culture wells. After 48 hr, responsiveness was measured by using a modified MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay.[31] One aliquot of SN was incubated in the absence of mAb, to test for IL-2 and IL-4 activities. A second aliquot was tested in the presence of anti-IL-2 mAb, for the determination of IL-4 activity, and a third aliquot in the presence of both anti-IL-2 and anti-IL-4 mAb. The detection threshold was 0·1 U/ml for IL-2 as well as for IL-4, as determined by standard titrations of recombinant lymphokines. One OD unit corresponded with an amount of 0·4–5 ng/ml of lymphokines, depending on the responsiveness of HT-2 cells in the individual bioassays. The level of cytokine production in the LD cultures ranged from 30 pg/ml to 2·5 ng/ml.

For detection of IFN-γ activity in SN from LD cultures, an ELISA was used.[26,32] The detection threshold was 2 U/ml, as determined by standard titration of recombinant IFN-γ.

RESULTS

Quantification of *L. major*-reactive T cells with the capacity to express granzyme A

Using immunohistochemistry as well as *in situ* hybridization, we have previously shown that numerous T cells expressing granzyme A can be detected in skin lesions and spleens of genetically susceptible BALB/c mice infected with *L. major*, whereas the numbers found in genetically resistant C57BL/6 mice are markedly lower.[9] To determine whether granzyme A is associated with $CD4^+$ or with $CD8^+$ T cells and whether it is expressed by *L. major*-reactive T cells, we now performed *in vitro* studies with selected T-cell populations. At various intervals after intradermal infection of BALB/c and C57BL/6 mice with *L. major*, spleen cells were collected and subjected to *in vitro* restimulation in bulk cultures followed by LD analysis.[26] For quantification of T cells reacting to *L. major*, parallel series of LD cultures were set up in the presence and absence of parasite antigen (see the Materials and Methods). After LD culture, SN was collected from each microwell for determination of lymphokine activity (see the following section), and the cells were lysed for subsequent performance of the enzyme assay.

An important finding was that *L. major*-reactive T cells expressing granzyme A *in vitro* could only be detected in lymphocyte populations obtained from infected BALB/c mice, but not in those from infected C57BL/6 mice. The frequencies of granzyme A-containing T cells from BALB/c mice are summarized in Table 1. The data show that significant numbers of both $CD4^+$ and $CD8^+$ T cells responding to *L. major* had the capacity to express granzyme A. Furthermore, it is evident that the frequencies of granzyme A^+ $CD4^+$ cells were generally higher than those of $CD8^+$ cells. This difference only disappeared at later stages of chronic disease when granzyme A activity became low for both T-cell populations. T cells from uninfected mice were consistently negative (data not shown).

Quantification of *L. major*-reactive T cells with the capacity to produce lymphokines

From each individual microwell that had been tested for enzyme activity, SN was collected for frequency determination of precursor T cells with the potential for lymphokine secretion. Because the aim of this study was to investigate whether granzyme A expression in *L. major*-infected mice correlates with lymphokine activity, only LD cultures set up with the granzyme A^+ T cells from BALB/c mice, but not those with the granzyme A^- cells from C57BL/6 mice, were analysed. The results in Table 2 show that considerable numbers of *L. major*-reactive $CD4^+$ T cells from infected BALB/c mice stimulated the proliferation of HT-2 cells, reflecting production of IL-2 as well as IL-4. The activity was reduced when the LD culture SN were assayed in the presence of anti-IL-2 mAb (Table 2), suggesting that it could partly be attributed to IL-2 production. The residual activity that had not been blocked by anti-IL-2 mAb reflected the release of IL-4, as confirmed by the finding that it was entirely inhibited when the SN were tested with the additional presence of anti-IL-4 mAb (data not shown). In accordance with previous studies in this and other laboratories, the frequencies of BALB/c T cells secreting IFN-γ were negligible at any time of infection (Table 2), whereas T cells from infected C57BL/6 mice produced high levels of IFN-γ.[1–4,6,26]

In the same LD system, $CD8^+$ cells from *L. major*-infected BALB/c mice showed only marginal levels of lymphokine production.[26] Therefore, it was not useful to include this T-cell

Table 1. Frequencies of *L. major*-reactive BALB/c precursor T cells with the potential of granzyme A expression*

	Reciprocal of precursor cell frequency (95% confidence limits)	
Weeks of infection	$CD4^+$ T cells	$CD8^+$ T cells
2	1199 (890–1835)	8925 (6245–15,847)
4	5302 (4512–6471)	27,128 (17,143–66,912)
6	44,464 (29,412–95,329)	19,631 (13,692–34,690)

*At various intervals after infection of mice with *L. major*, spleen cells were collected and subjected to a cycle of *in vitro* restimulation with *L. major* antigen followed by an interval of culture in the absence of antigen. Thereafter, limiting numbers of $CD4^+$ cells or $CD8^+$ cells selected by treatment with anti-CD8 mAb or anti-CD4 mAb, respectively, and complement were cultured with syngeneic accessory cells in the presence or absence of *L. major* antigen. LD cultures were restimulated 9 d later and, 24 hr thereafter, cells in each microwell were tested for granzyme A activity. Minimal estimates of the precursor frequency were obtained by the minimum χ^2 method. The data represent mean values from two experiments. All P values > 0·1.

Table 2. Frequencies of *L. major*-reactive BALB/c precursor $CD4^+$ T cells with the potential for secretion of IL-2, IL-4 or IFN-γ*

	Reciprocal of precursor cell frequency (95% confidence limits)		
	HT-2 response in the presence of		ELISA
Weeks of infection	No mAb IL-2 + IL-4	Anti-IL-2 mAb IL-4	IFN-γ
2	283 (220–395)	531 (408–761)	> 50,000
4	420 (314–632)	776 (538–1391)	> 50,000
6	404 (313–569)	ND	> 50,000

*See Table 1 for *in vitro* culture conditions. SN from individual cultures were collected and split for determination of lymphokine activities. IFN-γ was measured by ELISA. Both IL-2 and IL-4 were assessed by the proliferation of HT-2 cells in the absence of mAb; IL-4 activity was defined by the HT-2 response in the presence of anti-IL-2 mAb. All P values > 0·1.

ND, not determined.

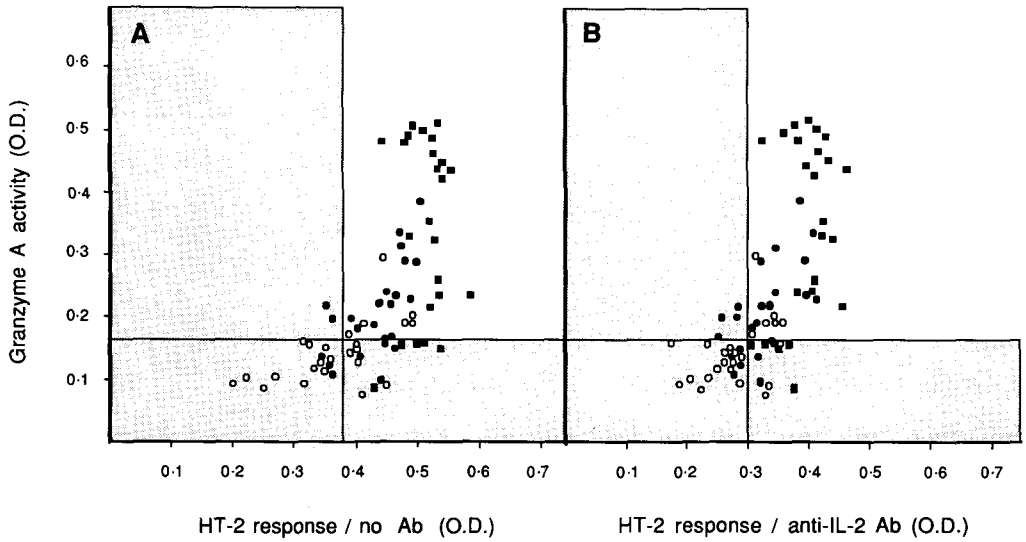

Figure 1. Comparison of granzyme A and lymphokine activities in LD cultures of $CD4^+$ cells obtained from BALB/c mice at 2 weeks of infection with *L. major*. Correlation of granzyme A expression with IL-2 as well as IL-4 production, as indicated by the HT-2 response in the absence of mAb (Ab) (A), and of granzyme A expression with IL-4 production, as indicated by the HT-2 response in the presence of anti-IL-2 mAb (B), in individual microcultures of T cells stimulated with *L. major* antigen. Twenty-four replicates were set up for each T-cell dose and the values are given for 156 (○), 312 (●) and 625 (■) cells/microwell, to ensure the clonal origin. The horizontal and vertical lines represent the mean absorbance values + 3 SD of control cultures containing accessory cells and antigen in the absence of T cells.

population in the analysis for co-ordinate expression of granzyme A and lymphokine activities.

Comparison of granzyme A and lymphokine activities in individual microcultures of LD assays

For the frequency determinations described above, granzyme A expression as well as IL-4 and/or IL-2 activities were monitored simultaneously in individual microwells at limiting cell numbers. It was therefore possible to analyse whether these functions were accomplished by the same or different populations of *L. major*-reactive T cells. Representative examples are depicted in Fig. 1 for 2 weeks of infection with *L. major*, and in Fig. 2 for 4 weeks of infection. The graphs clearly show that virtually all microcultures with granzyme A activity also promoted the growth of HT-2 cells. The addition of anti-IL-2 mAb diminished the HT-2 response, but the cultures were still positive, indicating residual IL-4 activity (Fig. 1B and 2B). Thus, expression of granzyme A by $CD4^+$ T cells from *L. major*-infected BALB/c mice correlated with secretion of the lymphokines IL-2 and IL-4. The co-existence of these activities was also observed with very low numbers of responder T cells, ensuring their clonal origin.

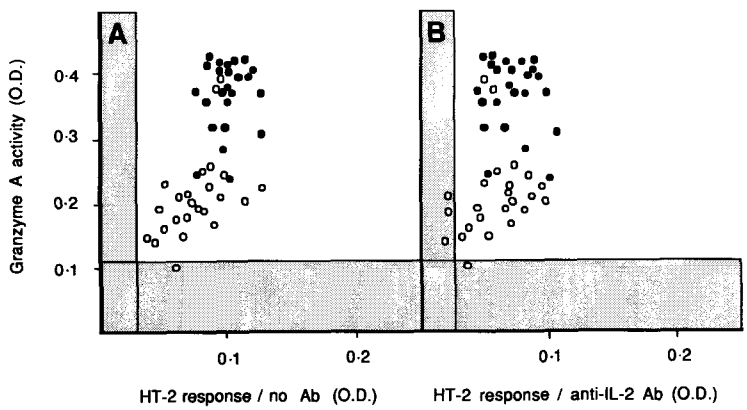

Figure 2. Comparison of granzyme A and lymphokine activities in LD cultures of $CD4^+$ cells obtained from BALB/c mice at 4 weeks of infection with *L. major*. Correlation of granzyme A expression with IL-2 as well as IL-4 production, as indicated by the HT-2 response in the absence of mAb (Ab) (A), and of granzyme A expression with IL-4 production, as indicated by the HT-2 response in the presence of anti-IL-2 mAb (B), in individual microcultures of T cells stimulated with *L. major* antigen. Twenty-four replicates were set up for each T-cell dose and the values are given for 625 (○) and 1250 (●) cells/microwell. See legend to Fig. 1.

DISCUSSION

We have previously documented that expression of granzyme A during murine *L. major* infection correlates with susceptibility to disease.[9] This was shown *in situ* using a mAb as well as a RNA probe specific for granzyme A to stain tissue sections. Because this finding indicated that granzyme A is associated with disease-promoting T cells, we have now performed *in vitro* analyses to characterize the subpopulation, the *L. major* responsiveness and the lymphokine production pattern of T cells expressing the enzyme. The results show that granzyme A activity in *L. major*-infected BALB/c mice can be assigned to parasite-reactive $CD4^+$ T cells producing IL-2 and IL-4, but not IFN-γ. Such a lymphokine secretion pattern is in agreement with our earlier finding that disease-promoting cells are different from Th2 cells. Since the LD system used for the present study allowed the functional analyses of T cells at the clonal level, it could be shown that the lymphokine activities and granzme A expression were elicited by individual populations of $CD4^+$ T cells.

On the basis of immunohistological methods, granzyme A-containing cells were detected in both susceptible BALB/c and resistant C57BL/6 mice infected with *L. major*, although the frequency of positive cells was significantly higher in BALB/c mice.[9] The present study revealed that *L. major*-reactive T cells expressing granzyme A could only be found in lymphocyte populations obtained from susceptible BALB/c mice. This finding suggests that the low number of granzyme A^+ cells previously observed in tissues of infected C57BL/6 mice reflected the presence of activated T cells with unrelated antigen specificity.

Although both T-cell subsets, $CD4^+$ and $CD8^+$, had the capacity to produce granzyme A in response to *L. major* infection, the frequency of $CD4^+$ T cells with enzyme activity was generally higher. This was observed *in vivo*[9] as well as in the present study *in vitro* and was particularly pronounced in the initial phase of infection when the highest numbers of granzyme A-containing T cells were detected. At later stages of chronic disease, the frequency of *L. major*-reactive $CD4^+$ T cells expressing granzyme A decreased continuously. On the other hand, the frequency of lymphokine-producing $CD4^+$ cells remained virtually constant.[26] Thus, although granzyme A activity and production of IL-2 and IL-4 are co-expressed by individual $CD4^+$ T-cell populations, the different time–course of those attributes indicates that they are regulated differently and that they fulfil distinct functions. While lymphokines have been shown to modulate macrophage activation[33,34] that is critical for elimination of the intracellular parasites, the protease granzyme A may be directly involved in tissue destruction and lesion formation.

This suggestion is supported by the capacity of granzyme A to degrade constituents of the extracellular matrix. Granzyme A has optimal activity under conditions of the extracellular space.[11] It has been shown to bind to and cleave the major glycosaminoglycan component of proteoglycans, heparan sulphate.[35] In addition, granzyme A is able to degrade the extracellular matrix molecules collagen type IV and fibronectin[36,37] and it may recruit additional proteolytic enzymes for accelerated disintegration of these structures.[38] By solubilizing subendothelial basement membranes, the cleavage of extracellular matrix proteins may facilitate the extravasation of T cells and other cell populations and may thus be involved in the completion of lesion formation. Such a situation is reminiscent of the graft rejection process. In the mouse and in biopsy material from transplant patients, the presence of granzyme A-expressing lymphocytes was found to correlate closely with the state of rejection.[39] In the latter case, most of the granzyme$^+$ T cells were $CD8^+$, whereas the experimental leishmaniasis model is an example for the *in vivo* role of granzyme A-containing $CD4^+$ cells.

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