Immune Depression Induced by *Trypanosoma cruzi* and Mouse Hepatitis Virus Type 3 Is Associated with Thymus Apoptosis

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*Trypanosoma cruzi*-infected mice show disturbance in the peripheral immune system such as polyclonal lymphocyte activation, autoantibody production, and immunosuppression of T lymphocytes. Previous observations in our laboratory showed that some stocks of *T. cruzi* can be contaminated with mouse hepatitis virus type 3 (MHV-3). Literature has shown that MHV-3 infection induces immunologic disorders characterized by thymic involution with marked cell depletion. However, the effects of interactions between MHV-3 and the parasite on the immune system are not well understood. In the present study specific-pathogen-free CBA mice were inoculated with MHV-3, alone or associated with different stocks of *T. cruzi*. Concurrent murine virus infection resulted in increased pathogenicity of *T. cruzi* infection shown by profound thymic atrophy; loss of cortical thymocytes; depletion of Thy1.2+, CD4+, and CD8+ cells; enhancement of in situ labeling of nuclear DNA fragmentation; and eventually, death of the animals. Such lines of evidence show that the mechanism underlying this thymic atrophy is associated with apoptosis. These results also suggest that MHV-3 can account for the increased immunosuppression observed during experimental infection with the parasite.

Chagas' disease, caused by the protozoan *Trypanosoma cruzi*, afflicts about 18 million people on the American continent (29). Although the disease has been extensively studied, its pathogenesis is far from being completely elucidated. Results obtained by using mice as an experimental model indicate that the outcome of infection is determined by many different factors. These include the genetic backgrounds of both the host (19, 26, 30) and the parasite (7, 18) and the experimental conditions under which infections are carried out (4). The role of concurrent infections in influencing the pathogenesis of *T. cruzi* infection resulted in increased pathogenicity of *T. cruzi* infection shown by profound thymic atrophy; loss of cortical thymocytes; depletion of Thy1.2+, CD4+, and CD8+ cells; enhancement of in situ labeling of nuclear DNA fragmentation; and eventually, death of the animals. Such lines of evidence show that the mechanism underlying this thymic atrophy is associated with apoptosis. These results also suggest that MHV-3 can account for the increased immunosuppression observed during experimental infection with the parasite.

**MATERIALS AND METHODS**

**Animals.** CBA/J (8- to 10-week-old) mice of both sexes were obtained from specific-pathogen-free colonies bred at Centro Multi-Institucional de Bioterismo, Unicamp. These colonies have been periodically screened since 1989 with consistently negative results for the following microorganisms: MHV-3, Sendai virus, lymphotic choriomeningitis virus, rotavirus, pneumonia virus of mice, reovirus 3, Thielers GD-VII virus, mouse minute virus, K virus, ectromelia virus, mouse adenovirus, mouse cytomegalovirus, and lactate dehydrogenase-elevating virus; *Mycoplasma pulmonis*; pathogenic bacteria; ectoparasites; and endoparasites. The animals were maintained in plastic isolators under aseptic conditions throughout this study. The stock of *T. cruzi* used in these experiments was originally received from Z. Brener in 1972 was labelled Y UEC and is maintained at the Department of Microbiology and Immunology, Instituto de Biologia/Unicamp, C.P. 6109, CEP 13083-970, Campinas, São Paulo, Brazil. E-mail: barsanti@obelix.unicamp.br.

**Parasites.** Two different stocks of the Y strain of *T. cruzi* were used. The stock originally received from Z. Brener in 1972 was labelled Y UEC and is maintained at the Department of Microbiology and Immunology, Instituto de Biologia/Unicamp, C.P. 6109, CEP 13083-970, Campinas, São Paulo, Brazil. E-mail: barsanti@obelix.unicamp.br.

**Protocols.** The animals were inoculated with MHV-3 isolated in our laboratory was used throughout the experiment. The stock Y UEC and is maintained at the Department of Microbiology and Immunology, Instituto de Biologia/Unicamp, C.P. 6109, CEP 13083-970, Campinas, São Paulo, Brazil. E-mail: barsanti@obelix.unicamp.br.

**Viruses.** MHV-3 isolated in our laboratory was used throughout the experiment. The stock Y UEC and is maintained at the Department of Microbiology and Immunology, Instituto de Biologia/Unicamp, C.P. 6109, CEP 13083-970, Campinas, São Paulo, Brazil. E-mail: barsanti@obelix.unicamp.br.

**Immunosuppression.** The mice were inoculated initially with MHV-3, alone or associated with different stocks of *T. cruzi*. Concurrent murine virus infection resulted in increased pathogenicity of *T. cruzi* infection shown by profound thymic atrophy; loss of cortical thymocytes; depletion of Thy1.2+, CD4+, and CD8+ cells; enhancement of in situ labeling of nuclear DNA fragmentation; and eventually, death of the animals. Such lines of evidence show that the mechanism underlying this thymic atrophy is associated with apoptosis. These results also suggest that MHV-3 can account for the increased immunosuppression observed during experimental infection with the parasite.

**Histology.** The thymi were removed, fixed in 10% formalin, and paraffin-embedded sections were stained with hematoxylin and eosin. The remaining thymi were cut into 10-mm-mm pieces and frozen in liquid nitrogen for subsequent RNA extraction and in situ DNA fragmentation assays.

**Immunohistochemistry.** The frozen sections were treated with a TdT-mediated dUTP nick end labeling (TUNEL) assay (23). The cells were stained with a biotinylated antibody as described previously (22).

**RNA extraction.** RNA was extracted from the frozen thymi using TRIzol reagent (GIBCO-BRL) according to the manufacturer's instructions. The RNA was treated with RNase-free DNase I (Promega) in the presence of the complete protease inhibitor cocktail P1 (Roche) to remove any contaminating DNA. The quality of the RNA was determined by spectrophotometry and by gel electrophoresis of RNA run on a 1.5% agarose gel. The RNA was stored at -80°C until use.

**In situ DNA fragmentation.** DNA fragmentation was assessed in situ by the TdT-mediated dUTP nick end labeling method (17, 20). The sections were incubated with TdT (Promega) in the presence of nuclease-p-free BSA and with a fluorescein-conjugated dUTP in 1× phosphate-buffered saline at 37°C for 1.5 h. The sections were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI), mounted in Prolong Antifade Mounting Medium (Molecular Probes), and examined with a Zeiss Axioskop microscope equipped with a 100× objective and a 100× Plan Neofluar objective. The degree of cell apoptosis was scored on a scale of 0 to 6, where 0 indicated no detectable DNA fragmentation and 6 indicated extensive DNA fragmentation throughout the entire thymus cortex and medulla. The slides were scored blind by two independent observers.

**Immunocompetence.** The thymic cellularity was measured in each group as the number of cells per mm of thymic tissue. The thymi were weighed and cut into 10-mm-mm pieces and frozen in liquid nitrogen for subsequent RNA extraction and in situ DNA fragmentation assays.

**Statistical analysis.** The data were analyzed by analysis of variance (ANOVA), unless otherwise indicated. A probability level of 0.05 was considered significant. The values of cellularity, DNA fragmentation, and thymus weight were expressed as means ± standard error of the mean (SEM). The Student's t-test was used to compare the differences between groups.

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FIG. 1. Thymic alterations during MHV-3, YCT, YUEC, and YCT–MHV-3 infection of animals. Weight (A) and cellularity (B) are dramatically diminished in groups infected with MHV-3, the YUEC stock of parasites, and YCT plus MHV-3 but remain stable in animals infected only with the YCT stock of parasites. The values for each day are means with standard deviations for experiments involving five mice analyzed individually. Normal levels in uninfected animals are delineated by horizontal lines.

### RESULTS

Thymus weight and cellularity were strikingly diminished in mice infected intraperitoneally with MHV-3, with the YUEC stock of *T. cruzi*, or with YCT plus MHV-3.

Figure 1A shows that the only group able to maintain thymic weight was that infected with the avirulent stock of parasites (YCT). All other groups that were infected with MHV-3, alone or associated with parasites, showed a marked decrease in thymic weight. The atrophy started 24 to 48 h p.i. and on day 7 p.i. represented approximately 50% of thymic weight in normal uninfected animals or the YCT-infected group.

As shown in Figure 1B, numbers of thymic cells decreased in MHV-3-infected animals as early as 24 h p.i. until day 3 p.i., when numbers showed some recovery. Moreover, these ani-

### Experimental design.
Four experimental groups of 25 CBA/J mice (total, 100 mice) were inoculated subcutaneously in the left hind limb with (i) 10⁶ trypomastigotes of the YCT stock of *T. cruzi*, (ii) 10⁵ trypomastigotes of the YUEC stock of *T. cruzi*, (iii) 0.1 LD₅₀ of coronavirus (MHV-3), and (iv) 10⁵ trypomastigotes of the YCT stock plus 0.1 LD₅₀ of MHV-3. Five mice in each group were sacrificed 1, 2, 3, 5, and 7 days post-inoculation, after which the thymuses were removed and weighed. Single-cell suspensions were obtained from these organs by gently teasing the tissue in RPMI 1640 medium. The cells thus obtained were washed twice and resuspended in the same medium, their viability was determined by the trypan blue exclusion test, and they were counted in a hemacytometer. Twenty-five mice were used as uninfected control group (normal group).

### Histopathological study.
At sacrifice, thymuses were collected and immediately fixed in buffered 10% formaldehyde fixative, and then they were routinely processed and embedded in paraffin. Sections (4 μm) for histological examination were stained with hematoxylin-eosin and examined by light microscopy.

### Lymphocyte subpopulation measurement.
Briefly, thymuses were individually collected after sacrifice, and cells were obtained by gently teasing with tweezers in RPMI 1640 medium. Cell suspensions were adjusted to give desired cell concentrations (2 × 10⁷/ml), laid in slides by centrifugation, air dried, fixed in cold acetone, and subjected to indirect immunoperoxidase assays. Endogenous peroxidase activity was abolished by immersing the slides for 20 min in methanol containing 0.3% H₂O₂. Different lymphocyte populations were identified by using a three-step staining procedure. First, the cells were incubated with monoclonal antibody (MAb) to T helper cells (GK 1.5 clone), MAb to T suppressor/cytotoxic cells (YTS 169 clone), or MAb to total T cells (Thy 1.2 antigen), followed by incubation with biotinylated goat anti-rat immunoglobulin G (Dako A/S) and finally with avidin-peroxidase complex (Vector Laboratories). The stain was developed by adding 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) solution containing 0.03% hydrogen peroxide and incubating for 20 min in darkness. The slides were weakly counterstained with Mayer’s hematoxylin (Merck) and examined by light microscopy. Cells showing positive staining were identified by the peripheral rim of brown color outlining the cell membrane. The percentage of positive cells was scored quantitatively by counting 100 cells in five microscopic fields and by two independent investigators.

### In situ identification of nuclear DNA fragmentation.
Tissue sections were analyzed by in situ end labeling of fragmented DNA as previously described (28). Briefly, after a 10% formaldehyde fixation, thymuses from infected and control mice were paraffin embedded. Sections were treated with proteinase K and finally with avidin-peroxidase complex (Vector Laboratories). The stain was developed by adding 3,3'-diaminobenzidine tetrahydrochloride and peroxidase (ApopTag-Oncor) were used to detect the reaction. Staining was developed in diaminobenzidine tetrahydrochloride solution and washed with phosphate-buffered saline, and residues of digoxigenin-nucleotide were catalytically added to the DNA by terminal deoxynucleotidyl transferase. Antidigoxigenin antibodies labelled with peroxidase (ApopTag-Oncor) were used to detect the reaction. Staining was developed in diaminobenzidine-H₂O₂, and sections were counterstained with Harris hematoxylin (Merck) and examined by light microscopy. Cells showing positive staining were identified by the peripheral rim of brown color outlining the cell membrane. The percentage of positive cells was scored quantitatively by counting 100 cells in five microscopic fields and by two independent investigators.

### Statistical analysis.
Student’s t test was used as described by Zar (31).

### RESULTS

| Group                  | CD4⁺ | CD8⁺ |
|------------------------|------|------|
| **Mean % ± SD (n = 5)**|      |      |
| Control                |      |      |
| YCT                    |      |      |
| YUEC                   |      |      |
| MHV-3                  |      |      |
| YCT + MHV-3            |      |      |

*Significantly different from the corresponding value for normal controls (P < 0.05).

### TABLE 1. Percentages of thymic cells expressing specific T-cell markers in parasite- and/or MHV-3-infected mice on days 1 and 7 p.i.

| Group                  | Thy 1.2⁺ | CD4⁺ | CD8⁺ |
|------------------------|----------|------|------|
| **Mean % ± SD (n = 5)**|          |      |      |
| Control                |          |      |      |
| YCT                    |          |      |      |
| YUEC                   |          |      |      |
| MHV-3                  |          |      |      |
| YCT + MHV-3            |          |      |      |

*Significantly different from the corresponding value for normal controls (P < 0.05).
mals were able to survive for more than 30 days after infection. Y_UEC– and Y_CT–MHV-3-infected mice showed thymic cell depletion around 48 and 72 h p.i., respectively, and numbers continued to fall until the animals died. No significant differences were observed in the numbers of thymic cells in Y_CT–infected animals. Virus infection, alone or in association with parasites, provoked significant decreases in the percentages of Thy1.2−, CD4+, and CD8+ cells in the thymus. This reduction in thymocyte subpopulations started at 24 h p.i. and reached maximum values on day 7 p.i. (Table 1).
FIG. 3. Apoptosis in a thymus section from a YCT-infected animal (A), a YCT plus MHV-3-infected animal (B), a MHV-3-infected animal (C), and a YUEC-infected animal (D). YCT-infected animals showed rare apoptosis granules, whereas the animals infected with the virus alone or in association with the parasite showed increased numbers of apoptotic cells. Hematoxylin counterstaining was used. Magnification, ×420.
Comparative histopathological studies of thymuses from stocks of T. cruzi- and MHV-3-infected mice showed differences in morphology and in the cortical/medullary content ratio. Thymus tissue from YCT-infected mice (Fig. 2A) was similar to that from control mice (not shown). In contrast, animals infected with YCT plus MHV-3 (Fig. 2B), MHV-3 alone (Fig. 2C), or Y UEC stock (Fig. 2D) showed significant thinning and cellular depletion in the thymic cortex and a decreased mitotic index (data not shown). Besides, these animals showed a “starry sky” pattern, caused probably by interspersed histiocytes.

In order to verify the origin of thymic atrophy and the fate of subpopulations of thymocytes, we investigated the degree of programmed cell death in tissue sections by in situ labelling of fragmented DNA. Y CT-infected mice (Fig. 3A) showed a round nuclear pattern, animals infected with YCT plus MHV-3 showed a few cells with fragmented DNA (Fig. 3A). In contrast, the number of apoptotic cells in the thymuses of mice infected with YCT plus MHV-3 (Fig. 3B) was dramatically increased. Thymuses of MHV-3-infected animals (Fig. 3C) and those infected with the Y UEC stock of parasites (Fig. 3D) showed significant numbers of apoptotic cells, but these numbers were lower than those in YCT-infected animals only recently infected with MHV-3 alone or with YCT-infected animals. In addition, different patterns of DNA fragmentation were observed in different groups. While animals infected with the Y CT or Y UEC stock of parasites (Fig. 3A and D) showed a round nuclear pattern, animals infected with YCT plus MHV or MHV alone (Fig. 3B and C) showed large clusters of irregular masses scattered in a disorganized cortico-medullary region.

**DISCUSSION**

Conventionally housed laboratory mice may sustain infections with one or more murine virus (10). The method of breeding mice under barrier-sustained conditions only recently became available to some laboratories, and so, earlier isolates of T. cruzi were maintained in mice that were coinfected by other pathogens.

Here we have observed a marked thymic cell depletion when animals were infected with MHV-3, alone or associated with one stock of the parasite. The diminished cellularity in thymus correlated well with decreased numbers of Thy1.2, CD4+, and CD8+ thymocyte subpopulations. These findings, showing that MHV-3 is able to exacerbate the parasite infection, together with the observed effects upon numbers of circulating lymphocytes (unpublished data) and thymic cells, suggest that the enhanced pathology associated with Y UEC infection reflects underlying alterations in the immune system. There are examples in the literature which show aggravation of both T. cruzi and murine leukemia virus by concomitant infections (25). It is known that MHV induces lymphoid organ atrophy (15) and shows a tropism to T and B lymphocytes (13). However, the mechanisms responsible for immunodeficiency associated with MHV-3 infection remain unknown.

The present results suggest that virus-induced programmed cell death could account for the loss of T lymphocytes from the thymuses observed after either Y CT plus MHV-3 or Y UEC infection. Together with the inhibition of thymocyte mitotic index in these animals, cellular death by apoptosis would be responsible for the thinning or atrophy of the thymus cortex. Apoptosis of T lymphocytes in animals infected with T. cruzi has already been demonstrated in spleen CD4+ cells (16). Similarly, several viruses, including some strains of MHV, are able to induce apoptosis (11, 21, 24). The unchanged numbers of both peripheral lymphocytes (unpublished data) and thymic cells, as well as the maintenance of a normal cortical/medullary content ratio in YCT-infected mice, are consistent with the low level of programmed cell death in this group. On the other hand, the more extensive apoptosis observed in the thymus in T. cruzi–plus MHV-3-infected mice suggests a severe immunosuppression which is probably triggered by the virus replication.

T-cell-dependent immune responses are crucial in the control of T. cruzi infection (2, 17, 23). Cellular immune responses mediated by helper and cytotoxic T lymphocytes are also involved in the elimination of viral infection (13). Therefore, control of both infections is dependent upon the capacity of the thymus to generate and maintain normal T lymphopoiesis.

In the present work we have observed a marked thymic involution in animals that were infected with the MHV-3 alone or with MHV-3 associated with T. cruzi, and this may well explain the greater pathology seen in these mice.

Several mechanisms including secretion of different mediators (1) could be responsible for the apoptosis seen during the course of the infections reported here. Further work is necessary to determine whether this apoptosis occurs as a result of direct lymphocyte or stromal cell infection or is caused by stress-induced glucocorticoid release and/or secretion of other soluble factors from intra- and extrathymic sites (12).

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