The Interaction between CD8+ Cytotoxic T Cells and *Leishmania*-infected Macrophages

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Summary

Leishmania is resident within the macrophages of its vertebrate host. In any intramacrophage infection, where the pathogen is present in a form capable of mediating cell to cell transmission, the contribution of a cytotoxic T cell response to protective immunity is questionable. This study presents data from an in vitro model designed to elucidate the outcome of an interaction between $CD8^+$, cytotoxic T cells and infected macrophages. Experiments were conducted with an H-2^d–restricted, cytotoxic $CD8^+$ T cell clone and *Leishmania* parasites present in mixed macrophage cultures, with the parasites confined to either histocompatible BALB/c macrophages, or incompatible CBA macrophages. Initial experiments indicated that the viability of *Leishmania* was unaffected by the lysis of its host macrophage by cytotoxic T cells. However, extended experiments showed that the parasites were killed between 24 and 72 h. The same results were obtained regardless of whether the parasites were resident in the target, BALB/c, macrophages or the bystander, CBA, macrophages. Addition of neutralizing, anti–IFN-g antibody to the cultures ablated most of the leishmanicidal behavior, indicating that parasite death was attributable to macrophage activation, resulting from cytokine secretion from the T cells following the initial recognition event.

The pathology of all diseases induced by intracellular pathogens is the product of a complex series of interactions between the pathogen and its host cell, and the infected host cell and the immune system. Indeed, under certain conditions, the immune system appears responsible for the exacerbation and increased morbidity of an infection (1–3). One interaction of particular interest in intracellular infections is that between the cytotoxic T cell and a pathogen-infested macrophage. If the pathogen exists in an infective form, capable of mediating cell to cell transmission, then the value of such an immune response is open to question, as discussed by Kaufmann for *Mycobacterium* (4). This interaction has three possible outcomes. First, both the pathogen and host cell could be killed in the cytotoxic event. Second, the pathogen could be released, spreading the infection. And, finally, the pathogen could be released to infect naive "bystander" macrophages in the vicinity, which are in turn activated to kill the pathogen by cytokines released by the cytotoxic T cell stimulated during the original target/host cell cognitive event.

The intracellular protozoan parasite *Leishmania* represents an excellent model for dissecting out these potential microbicidal mechanisms. *Leishmania* exists as an amastigote within the macrophages of its vertebrate host (5, 6). The macrophage expresses class I antigen and is a target for $CD8^+$ cytotoxic T cells, and the amastigote form of *Leishmania* is infective following release from its host cell. In addition, macrophages activated by cytokines, such as IFN-g, show an elevated level of leishmanicidal behavior (7, 8). In vitro *Leishmania*–infected macrophages can be sensitized with synthetic peptide epitopes of characterized cytotoxic T cell clones, rendering them targets for class I-restricted cytotoxic attack. The use of cocultured macrophages of noncompatible class I haplotype provides fresh host cells for released parasites, and facilitates direct examination of "bystander" killing mechanisms. These noncompatible macrophages serve as a model for intralesional macrophages that are either in the vicinity of infected macrophages, but are not in contact with the T cells; or macrophages that are not presenting relevant antigen. The *Leishmania*-infected macrophage offers a uniquely accessible in vitro model system for studying the potential outcome of the interaction between cytotoxic T cells and infected host cells.

The in vivo role of $CD8^+$ cytotoxic T cells in leishmaniasis is unclear. The majority of research into protective immunity against leishmania has dealt with the IFN-g–producing, TH1 helper cell subset (9), which would be stimulated through antigen presentation in context with class II MHC. *Leishmania* amastigotes remain within the host cell's

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lysosomal system (10–13) while class I-associated antigen presentation, required for stimulation of CD8 lymphocytes, is usually restricted to antigens of cytoplasmic origin. There is, however, an increasing body of evidence implicating a $CD8^+$ T cell response in the resolution of murine experimental leishmaniasis (14–16). This present study does not broach this issue, but confines itself to examining microbicidal events triggered by recognition of infected macrophages by cytotoxic T cells.

Materials and Methods

Cell Culture. Promastigotes of *Leishmania mexicana mexicana* (MNYC/BZ/62/M379) were maintained in SDM79 medium at 25°C. Macrophages for infection with *Leishmania* were isolated by peritoneal lavage from female CBA or BALB/c mice. The H-2^d restricted, $CD8^+$, cytotoxic T cell clone CS.B35 specific for the circumsporozoite protein of *Plasmodium berghei* (17, 18) was maintained in DMEM-high glucose supplemented with 0.2 g/l L-arginine, 0.036 g/l L-asparagine, 10 mM Hepes, 2 mM L-glutamine, 50 μM 2-ME, 10% FCS (Hyclone Laboratories, Logan, Utah) and either 1.5–2% T cell growth factor, derived from phorbol myristate-activated EL-4 cells, or 10% CR-Rat T-stim without con A (Collaborative Research, Bedford, MA). Cells were maintained by weekly restimulation of clones (0.125×10^6/ml) with irradiated feeder cells (2.5×10^6/ml), and irradiated P815 cells (1.25×10^6/ml) pulsed with 1 μM of synthetic peptide (amino acids 249-260 of the *P. berghei* CSP sequence). 4 d later, cultures were split and grown for a further 3 d, or used in cytotoxicity assays.

Cytotoxicity Assays. The ability of the cytotoxic T cell line CS.B35 to lyse target cells expressing class I of the correct genetic haplotype following sensitization with synthetic peptide sequence (Pb.249-260) was monitored initially by release of ^{51}Cr, as described (19). The % specific lysis was expressed as a product of the sample release, minus spontaneous release, divided by detergent release, minus spontaneous release. Following calculation of the required effector to target cell ratio, effective cytotoxic T cell stimulation was confirmed by release of serine esterase into the medium (20). 1 h after the addition of T cells to the experimental wells, 20 μl of medium was removed and mixed with benzylocarbonyl-l-Lysine thiobenzyl ester (0.2 mM) and dithiobis (2-nitrobenzoic acid) (0.2 mM) in PBS. Absorption at 412 nm was determined with microtitre plate reader. Specificity was controlled by incubation in the presence of noncompatible or nonsensitized target cells.

Leishmania Killing Assays. The survival of intracellular *Leishmania* following attack on their host cell by cytotoxic T cells was evaluated by the experimental conditions summarized in Fig. 1. Residential macrophages from either CBA or BALB/c mice were isolated by peritoneal lavage, adjusted to 10^5 cells/ml, and plated as 1 ml in 12-well plates, all experiments were conducted with triplicate wells. Following 24 h the cells were infected with a 3:1 excess of stationary phase *L. mexicana* promastigotes, left for 2 h and then washed to remove any residual parasites. After 48 h an equivalent number of uninfected macrophages of the alternative mouse strain (CBA or BALB/c) were then added. Cultures were then left for a further 3 d before use in T cell interaction experiments. In this way cultures were established whereby parasites were restricted to either the bystander (CBA, H-2^k), Fig. 1 *a*, or target (BALB/c, H-2^d), Fig. 1 *b*, macrophage haplotype. *Leishmania* survival experiments were carried out by sensitizing the mixed macrophage cultures with CSP peptide and adding the cytotoxic T cell clone CS.B35

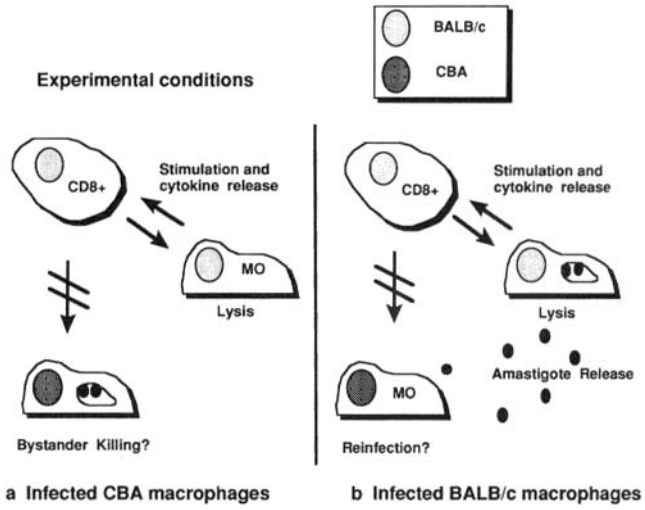


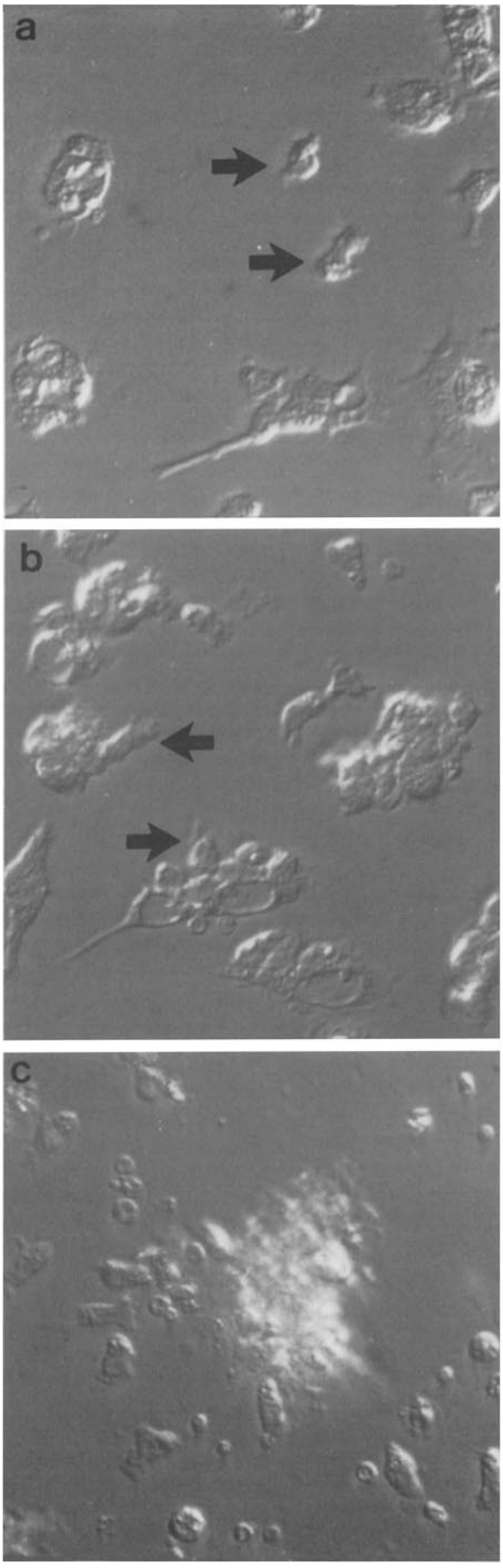
Figure 1. Diagrammatic representation of the experimental culture conditions used to determine *Leishmania* viability following the interaction with cytotoxic T cells. *Leishmania* amastigotes were established in CBA (*a*) or BALB/c (*b*) macrophages. 2 d after infection an equal number of uninfected macrophages, (*a*) BALB/c, (*b*) CBA, were added. 3 d later, all macrophages were sensitized with synthetic peptide and a 3–4:1 excess of BALB/c, $CD8^+$ cytotoxic T cells were added. In well (*a*), the T cells will recognize and lyse the uninfected BALB/c macrophages, facilitating evaluation of "bystander" killing in the infected CBA macrophages. In well (*b*), the T cells will recognize and lyse the infected BALB/c macrophages, allowing evaluation of the leishmanicidal activity of the cytolytic event, and the reinfection capabilities of the released amastigotes. Long term experiments should reveal whether cytokines released by the $CD8^+$ cytotoxic T cell, stimulated by the initial recognition event, have any effect on ultimate parasite survival.

in varying ratios for various lengths of time. The relative parasite survival was calculated as follows. Initially, incorporation of ^{3}[H]-uracil, which is used only by the parasite, was employed as a measure of parasite viability, however, the length of incubation required to achieve levels of incorporation significantly above background rendered time point experiments difficult to interpret. Instead, wells were scraped to remove all cells, passaged through a 25 gauge needle, and resuspended in 1 ml of the promastigote medium SDM79 at 25°C for 20 h. The medium was subsequently pipetted up and down to ensure homogeneity, and a 50 μl sample was plated on agar plates consisting of SDM79 mixed with Blood Agar Base No2 (Oxoid) to a final agar concentration of 1%. Plates were incubated at 25°C for 10 d, and scored by counting 5 independent 1 cm^2 areas. Plates were prepared from each of the triplicate wells.

Anti-IFN-g Inhibition of Parasite Killing. The contribution of IFN-g secretion from stimulated T cells to the killing of *Leishmania* was assessed by the addition of the anti-IFN-g hamster mAb, H22 (21), at 25 μg/ml. Control wells received an equivalent amount of irrelevant hamster mAb. Antibodies were added to all wells every 24 h throughout the duration of the experiment, starting from the time of addition of T cells to the macrophage monolayers.

Results

Cytotoxicity of CS.B35 Anti-CSP $CD8^+$ Clone on Sensitized BALB/c Peritoneal Macrophages. The ability of H-2^d restricted cytotoxic T cell clone CS.B35 to lyse BALB/c mac-



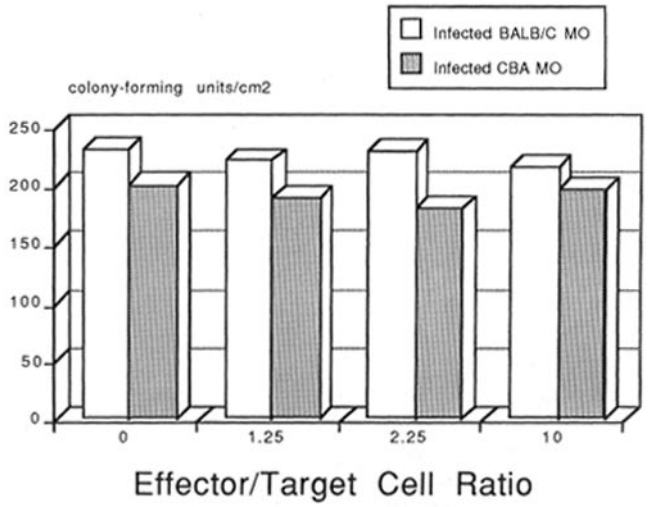


Figure 3. Effects of host cell lysis on *Leishmania* viability. A graph illustrating the viability of *Leishmania mexicana* recovered from mixed cultures of infected CBA and uninfected BALB/c and uninfected CBA macrophages. Within a short term assay of 2 h duration, at varying ratios of E/T cell, a comparable number of viable parasites was recovered regardless of the class I haplotype of the infected macrophage. The stable nature of the parasite number despite the macrophage haplotype and the variation in the effector to target cell ratio indicated that *Leishmania* viability was unaffected by lysis of their host cell by cytotoxic T cell attack. Viability was scored by the relative colony-forming abilities of *Leishmania* recovered from the wells. Lysis of target macrophages was confirmed by light microscopy. Experiments were performed in triplicate and comparable results were obtained in two independent experiments.

rophages following sensitization with synthetic peptide was assayed by release of cytoplasmic ^{51}Cr from sensitized target cells. Within a 1 h coincubation period the level of radioactivity release achieved with an E/T cell ratio exceeding 2.5:1 equaled that of the detergent-treated controls, indicating that cell lysis had reached an end point (not shown). No lysis was detected when the T cells were incubated with either BALB/c macrophages that had not been sensitized with peptide, or with sensitized CBA macrophages, demonstrating the genetic and antigenic restriction of cell lysis. Following this initial calculation of required E/T cell ratio, the efficacy of cytotoxic T cell stimulation was assayed by serine esterase release and light microscopical examination of the culture wells. Fig. 2 shows a series of light micrographs of CS.B35 T cells interacting with *Leishmania*-infected CBA macrophages (*a*), and with sensitized, infected BALB/c macrophages, (*b* and *c*).

Survival of Intracellular Leishmania Amastigotes During Cytotoxic Attack on the Host Cell. This series of experiments was designed to determine the effect of a cytotoxic T cell attack

Figure 2. Hoffman Modulation Contrast light micrographs of *Leishmania mexicana*-infected macrophages and the cytotoxic T cell clone CS.B35. (*a*) CBA macrophages following sensitization with peptide and addition of cytotoxic T cells. There is little interaction between the cytotoxic T cells (*arrowed*) and these macrophages that display an incompatible class I antigen haplotype. (*b* and *c*) BALB/c macrophages following sensitization with peptide and addition of cytotoxic T cells. The T cells (*arrowed*) quickly associate with the surface of the macrophages (*b*, 20 min), and within 1 h the macrophages have been completely lysed (*c*).

the host macrophage. Both BALB/c and CBA macrophages were independently infected with *Leishmania* and left for 24 h for the infection to become established. An equal number of macrophages from the alternate mouse strain, and genetic background, were then added to the wells which were incubated for a further 3 d before use in experiments, see Fig. 1 for summary. In the initial experiment, macrophages in all wells were sensitized with synthetic peptide, washed, and then incubated for 2 h with varying numbers of effector, CS.B35 clone T cells. Light microscopical examination of the wells after 2 h supported the data generated in the preceding experiment that an E/T cell ratio exceeding 2:1 resulted in complete lysis of relevant target cells. *Leishmania* were recovered from the various wells by disruption of cells. The parasites were then left to differentiate for 20 h and plated onto nutrient agar. Colonies were scored 10 d later, reported in Fig. 3. A comparable and constant number of colonies were produced, irrespective of whether the parasite was residing in the susceptible, BALB/c macrophage, the target cell for lysis, or in the bystander CBA macrophage. These results indicate that the viability of the intramacrophage-*Leishmania* amastigote is unaffected by the destruction of its host cell by the cytotoxic T cells. The inclusion of a set of wells without T cells, and the use of infected bystander CBA macrophages acted as controls for possible processing artefacts.

Long-term Viability of Leishmania Following Lysis of Host Macrophages. The preceding experiment demonstrates that *Leishmania* is released in a viable condition following lysis of its host macrophage. However, the release of viable parasites does not necessarily guarantee successful spread of infection, especially if the recipient host cells have been activated by cytokine release from the cytotoxic T cells. Fig. 4 shows the result from an extended incubation of CS.B35 cytotoxic T cells with similar mixed infected BALB/c and uninfected CBA macrophages, or infected CBA and uninfected BALB/c macrophages. The ratio of effector to target cells was maintained at 3–4:1 for these experiments. Samples taken 2 h and 24 h following addition of the cytotoxic T cells show little change in the number of viable parasites recovered from the wells. However, samples taken at 48 h and 72 h time points showed an almost complete eradication of the parasite population. Light microscopical examination of these cultures indicated that approximately 50% of the original mixed macrophage population persisted, apparently unaffected by the conflagration. Control wells confirmed that T cell stimulation and macrophage lysis were restricted to BALB/c macrophages. Interestingly, *Leishmania* resident within the bystander CBA macrophages were killed at a rate comparable to those within the target, BALB/c macrophages.

Verification of the bystander-killing effect induced by stimulated $CD8^+$ cytotoxic T cells is shown in Fig. 5. In this experiment infected CBA macrophages were mixed with uninfected BALB/c, target macrophages. Half the wells were sensitized with the relevant peptide, and the cytotoxic clone CS.B35 was added, at a 3–4:1 ratio to BALB/c macrophages, to all wells. In the wells where no peptide had been added to sensitize the BALB/c macrophages, the *Leishmania* amastigotes increased in number within their CBA host macrophages. Serine esterase assays confirmed that no T cell stimulation had occurred in these wells. In contrast, in the wells

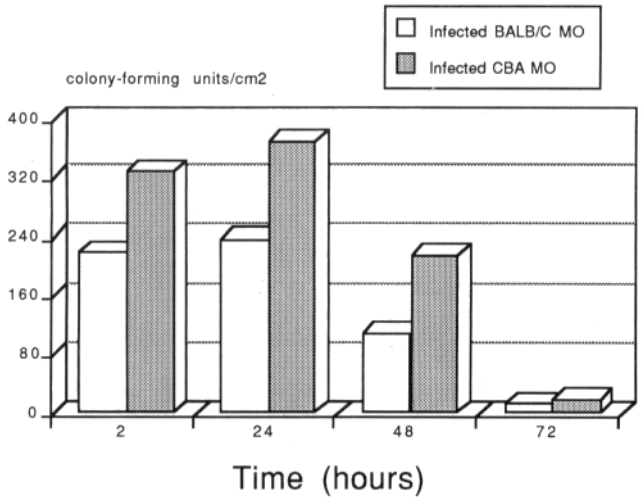


Figure 4. Long-term effects of host cell lysis on *Leishmania* viability. A graph illustrating the viability of *Leishmania mexicana* recovered from mixed cultures of infected CBA and uninfected BALB/c macrophages or infected BALB/c and uninfected CBA macrophages. In a longer term assay of 72 h duration, the viability of *Leishmania* was strongly inhibited regardless of whether the parasite was situated within the target BALB/c macrophages, or bystander CBA macrophages. Viability was scored by the relative colony-forming abilities of *Leishmania* recovered from the wells. Lysis of target macrophages was confirmed by light microscopy. Experiments were performed in triplicate and comparable results were obtained in two independent experiments.

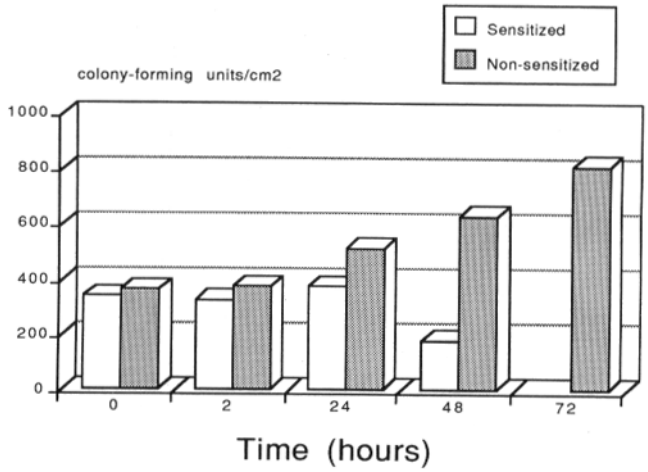


Figure 5. Killing of *Leishmania* in Bystander macrophages is dependent on an effective interaction between cytotoxic T cells and uninfected BALB/c macrophages. A graph of the survival of *Leishmania* in CBA macrophages in the presence of CS.B35 T cells and sensitized and control BALB/c macrophages. Parasite number increased against time in the control wells, while wells containing sensitized BALB/c macrophages showed a strong leishmanicidal activity from 24 h onwards. Viability was scored by the relative colony-forming abilities of *Leishmania* recovered from the wells. Lysis of target macrophages was confirmed by light microscopy. This experiment was performed in triplicate.

containing cells sensitized with peptide, the intracellular *Leishmania* were killed despite the fact that the BALB/c macrophages were the target cells. These wells contained serine esterase activity indicating stimulation of the cytotoxic T cells.

Suppression of Anti-Leishmania Killing by Addition of Anti-IFN-g. The most obvious mechanism by which *Leishmania* amastigotes in bystander macrophages may be killed by a cytotoxic T cell lysing a neighboring cell would be through macrophage-activation by release of cytokines from the stimulated T cell. To test this possibility, mixed CBA and BALB/c, *Leishmania*-infected, and uninfected macrophage cultures, were prepared as described and shown in Fig. 1. Cultures were sensitized with peptide and incubated with CS.B35 cytotoxic T cells. An IFN-g–neutralizing mAb was added at time of addition of T cells, and every 24 h. An equivalent amount of irrelevant antibody was added to the control wells. As seen in Fig. 6, the addition of anti–IFN-g antibody suppressed the leishmanicidal activity in the cultures. This effect was more marked in the infected-CBA macrophage cultures, although the *Leishmania* parasites in both cultures failed to expand to the level found in the preceding experiment, Fig. 5.

Discussion

In this study *Leishmania*-infected macrophages were used as targets for cytotoxic T cell attack to determine the potential efficacy of a cytotoxic T cell response to a pathogen that exists in an infective form within its host cell. The results of this study have relevance for a considerable number of bacterial (*Salmonella*, *Listeria*, *Mycobacteria*, *Coxiella*), protozoal (*Leishmania*, *Trypanosoma cruzi*), and fungal (*Histoplasma*) intracellular pathogens. The role of cytotoxic T cells in mycobacterial infections has already been the subject of detailed reviews (4, 22).

Results from the initial, shortterm experiments demonstrate that the lytic attack, which causes destruction of the host macrophage, does not result in the direct death of the *Leishmania* amastigotes. Parasite viability was measured by a CFU assay, and the viable parasite yield was comparable irrespective of whether the amastigotes were in the target macrophages (H-2^d) or the cocultured, incompatible-class I haplotype "bystander" macrophages (H-2^k). However, when the length of incubation time was extended, *Leishmania* amastigotes were killed from 24 h onwards. At 72 h parasite

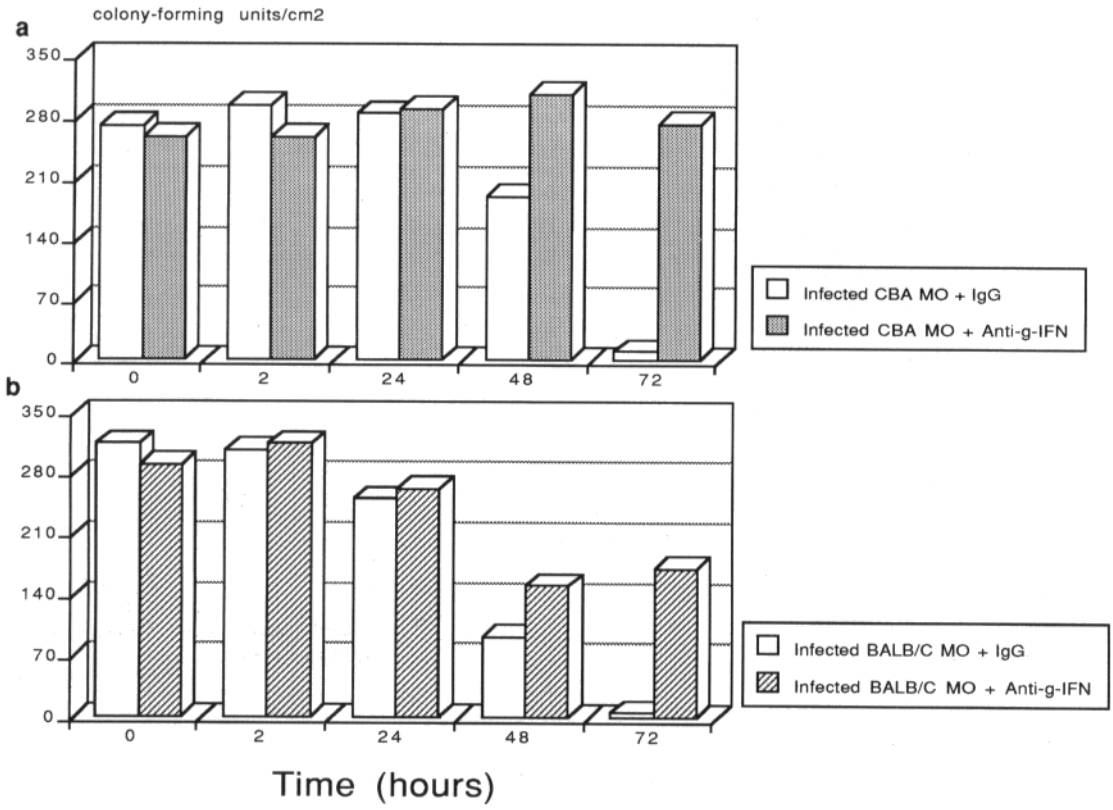


Figure 6. Anti–IFN-g antibody blocks the leishmanicidal activity. These graphs show the numbers of viable *Leishmania* recovered from mixed macrophage infections following interaction with cytotoxic T cells in the presence of anti–IFN-g or control antibody. Both antibodies were added at 25 μg/ml at time 0 h, 24 h, and 48 h. (*a*) The killing of *Leishmania* in infected bystander CBA macrophages was almost totally inhibited by anti–IFN-g antibody, although the parasites did not increase in number to the extent found in earlier experiments, Fig. 5. (*b*) The killing of *Leishmania* in the target BALB/c macrophages was blocked to a slightly lesser degree by anti–IFN-g antibody. Viability was scored by the relative colony-forming abilities of *Leishmania* recovered from the wells. Lysis of target macrophages was confirmed by light microscopy. Experiments were performed in triplicate and comparable results were obtained in two independent experiments.

death exceeded 94%. Again this effect was irrespective of the haplotype of the host macrophages. To further examine this bystander-killing effect, cytotoxic T cells were added to sensitized or non-sensitized cultures of infected CBA macrophages and uninfected BALB/c macrophages. In the nonsensitized controls the parasites doubled in number, while in the sensitized cultures they were killed although the host macrophages were not themselves subject to lytic attack.

Experimental data from both in vitro and in vivo studies strongly suggest that immune clearance of *Leishmania* is primarily due to IFN-g–producing T cells that induce a state of activation in parasitized macrophages and, ultimately, death of the parasite by generation of toxic nitrites (7, 8, 23, 24). To examine a role for macrophage-activation in this present study, an IFN-g–neutralizing antibody was added to the infected macrophage cultures at the same time as the cytotoxic T cells. The antibody strongly suppressed the ability of the cultures to kill the infecting *Leishmania*. In the cultures with infected-CBA macrophages, in the presence of neutralizing antibody, the parasite number remained relatively constant, 105% of the starting number, whilst in the control only 4% of the parasites survived. In the infected BALB/c cultures a similar level of killing was achieved in the irrelevant antibody control, however, the neutralizing antibody containing cultures yielded only 59% of the original parasite load. The difference between the two anti–IFN-g–containing cultures likely represents the reinfection efficiency of the *Leishmania* amastigotes released by the lysis of the infected BALB/c macrophages. This interpretation is supported by microscopical examination of the cultures at 72 h which revealed that the majority of intact macrophages, presumably the CBA bystander macrophages, contained amastigotes that must have originated from infected BALB/c macrophages. Finally, although a marked inhibition is achieved with neutralizing antibody against IFN-g, it does not rule out the participation of other cytokines, such as tumor-necrosis factor (23), working in synergy in the induction of microbicidal responses.

The existence of $CD8^+$ cytotoxic T cells in a leishmanial infection is a point of some controversy. The *Leishmania* amastigote exists within a membrane bound vesicle of endosomal/lysosomal origin (13). Antigen presentation from this intracellular compartment would, by convention, be expected to proceed through complexing with class II MHC molecules and stimulation of T cells of the $CD4^+$ phenotype (9). Recently, however, data has been accumulating that suggests that *Leishmania* infection also induces a $CD8^+$ T cell response, and that this response contributes to the resolution of the murine disease (14–16). *Mycobacteria* also remain within membrane bound compartments of their host macrophages, however, CTL responses to mycobacterial proteins have been found in experimental murine infections (22). And, most recently, another intravacuolar pathogen, *Salmonella*, engineered to express the *Plasmodium* circumsporozoite protein, induced a $CD8^+$, cytotoxic T lymphocyte response (25). These results indicate that antigens from at least some intravacuolar pathogens gain access to a class I-antigen presentation pathway.

Irrespective of the dichotomy concerning class I antigen presentation, the nature of the interaction between $CD8^+$ cytotoxic T cells and infected macrophages is one of considerable interest for all intramacrophage infections. This present study has exploited an artificial in vitro model to study the outcome of an interaction between $CD8^+$ cytotoxic T cells and *Leishmania*-infected macrophages. The results indicate that pathogens that exist within their host cell in a form capable of mediating transmission, may be released in a viable state by the activity of a cytotoxic T cell response. However, if the site of infection is contained, such as within a cutaneous *Leishmania* lesion, the release of cytokines, like IFN-g, could lead to activation of bystander macrophages and ultimate death of the microbes. These results further underline the desirability of an immune response which leads to expansion of IFN-g–producing T cells, whether they be $CD4^+$, TH1, or $CD8^+$.

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