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Transfer of Protective Immunity in Murine Histoplasmosis by a CD4$^+$ T-Cell Clone

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We have reported that a murine *Histoplasma capsulatum*-reactive CD4$^+$ T-cell line and clones thereof did not adoptively transfer protection against *H. capsulatum* infection in normal or cyclophosphamide-treated C57BL/6 mice. One explanation for the results was that the T cells failed to traffic to lymphoid organs in these animals. In this study, we have sought to determine whether one of these clones, 2.3H3, could mediate protection in nude (C57BL/10) or irradiated (5 Gy) heterozygous nude (*nu*/+) C57BL/6 mice. Mice were inoculated intravenously with 10^7 resting 2.3H3 cells or with an equal number of cells of the ovalbumin-reactive clone 1S6; 2 h later, the mice were challenged intranasally with 5×10^6 yeast cells. By day 5 of infection, lungs, livers, and spleens of nude and irradiated *nu*/+ mice given 2.3H3 contained significantly fewer ($P < 0.05$) CFU than the same organs from mice inoculated with 1S6. This effect was specific for *H. capsulatum*, since 2.3H3 did not reduce the number of *Coccidioides immitis* CFU in lungs, livers, and spleens of irradiated *nu*/+ mice. By day 10, the amounts of *H. capsulatum* CFU in lungs, livers, or spleens of nude and irradiated *nu*/+ mice inoculated with 2.3H3 were smaller than those in 1S6-inoculated mice, but these differences did not reach statistical significance ($P > 0.05$). The mortality rate of mice inoculated with 2.3H3 and that of mice inoculated with 1S6 were similar. Histopathological examination of tissues from 2.3H3- and 1S6-inoculated mice demonstrated the presence of granulomatous inflammation in organs from both groups. Tissues from 2.3H3-treated mice contained fewer yeasts per high-power field than tissues from 1S6-treated mice. Thus, irradiated or nude mice are permissive for the expression of protective immunity by a CD4$^+$ T-cell clone. Although the protective capacity of T cells in these animals is transient, these animals will be useful for differentiating protective from nonprotective T-cell clones.

Histoplasma capsulatum is a dimorphic fungal pathogen that produces a broad spectrum of disease ranging from respiratory illness to a disseminated form (5, 9, 11). Most often, infection is self-limited and does not require therapeutic intervention. An effective host response is dependent on interaction between antigen-reactive T lymphocytes and macrophages (10, 12, 13). In particular, the CD4$^+$ subset plays a central role in the elimination of *H. capsulatum* yeast cells (2, 4). The development of *H. capsulatum*-reactive murine T-cell clones has provided a tool for analyzing the role of T cells in antifungal immunity. These cells, which are CD3$^+$ CD4$^+$, mediate local delayed-type hypersensitivity when injected with antigen into footpads (3). The cells exert several functions in vitro, including release of interleukin 2 and release of a factor which enables macrophages to limit growth of *Histoplasma* yeasts (3).

However, little is known about the immunological functions of these T cells in vivo. In a previous study, systemic transfer of a CD4$^+$ murine T-cell line (JC1) and three T-cell clones (2.3H3, 2.3E10, and 1.3G6) failed to decrease the number of CFU in spleens of naive and cyclophosphamide-treated C57BL/6 mice after intravenous (i.v.) infection with *Histoplasma* yeast cells (2).

The present study examined whether clone 2.3H3 could protect immunoimpaired mice after intranasal infection with *H. capsulatum* and whether protection would be specific to this fungus.

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MATERIALS AND METHODS

Animals. Female C57BL/6 (*H-2^b*) *nu*/+ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Female and male C57BL/10 (*H-2^b*) nude and heterozygous *nu*/+ mice were obtained from our own breeding colony.

Pathogens. *H. capsulatum* G 217 B was maintained in stock cultures on Sabouraud dextrose agar at 37°C. For infection, yeasts were grown in brain heart infusion broth supplemented with 1% culture filtrate (filter-sterilized supernatant of 30-day cultures of *H. capsulatum* grown in brain heart infusion broth at 37°C in a shaker incubator) at 37°C in a shaker incubator (New Brunswick) at a gyratory speed of 150 rpm. After 36 h, the cells were harvested and washed three times in saline. Viability was then determined by fluorescein diacetate-ethidium bromide (FDA-EB) staining. Briefly, stock solutions of FDA were prepared in acetone at a concentration of 5 mg/ml and kept at −20°C. EB was dissolved in phosphate-buffered saline (PBS) at a concentration of 50 μg/ml. Just before use, the FDA solution was diluted 1:2,500 in PBS and mixed with an equal volume of EB solution. Next, equal volumes of FDA-EB solution and yeast suspensions were mixed and incubated at 37°C for 30 min. One drop of suspension was then placed on a slide, covered with a coverslip, and examined under a fluorescence microscope. One hundred cells were counted, and the percentage of cells that were dead (red) was determined. Next, yeasts were counted on a hemacytometer and adjusted to the desired concentration (10^8/ml) in saline; this concentration was confirmed by quantitative culture.

Coccidioides immitis C 735 was maintained on glucose-yeast extract agar at 25°C. Arthroconidia were harvested

from well-sporulated cultures by placing a magnetic bar on top of the culture plate, overlaying the plate with PBS, and spinning the bar, thus breaking up the mycelium. The arthroconidia were washed three times in saline and counted on a hemacytometer. Arthroconidia were adjusted to 8×10^2/ml, and the concentration was confirmed by quantitative culture.

Antigens. Histoplasmin was prepared from mycelial-phase cultures of *H. capsulatum* grown in Smith's asparagine medium (3). Prior to use, it was dialyzed against PBS, pH 7.4, for 24 h. Ovalbumin (catalog no. A 2512) was obtained from Sigma Chemical Co., St. Louis, Mo.

Propagation of T-cell clones. Clones 2.3H3 (histoplasmin reactive) and 1S6 (ovalbumin reactive) were maintained and expanded in 24-well plates in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS) (HyClone), 1% nonessential amino acids, 2% L-glutamine, 1% sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Wells containing 2×10^5 viable cells per ml were stimulated every 10 days with 2×10^6 irradiated (30 Gy) splenocytes, 0.2 μg of histoplasmin (2.3H3) per ml or 250 μg of ovalbumin (1S6) per ml, and 20 U of human recombinant interleukin 2 (Genentech) per ml.

Systemic transfer and induction of infection. Twenty-four hours before transfer, heterozygous *nu*/+ mice were irradiated with 5 Gy each. On the day of infection, resting T cells were centrifuged over Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada), washed twice, and suspended in RPMI 1640 plus 5% FBS. They were adjusted to 2.5×10^7 viable cells per ml, and each mouse (6 to 10 mice per group) received 10^7 1S6 or 2.3H3 cells in 0.4 ml i.v. via the lateral tail vein. Two hours later, the mice were anesthesized intraperitoneally with pentobarbitol sodium (Nembutal; 50 mg/kg of body weight) and infected intranasally with 5×10^6 cells of *H. capsulatum* or (for experiments with *C. immitis*) with 40 arthroconidia in a volume of 0.05 ml. Deaths within 48 h of infection were not included in the results. For mortality studies, groups of 6 to 10 mice received either clone 2.3H3 or 1S6 or cell-free RPMI 1640. Mice were infected and then observed daily for mortality. On days 5 and 10 of infection, target organs were removed, weighed, and homogenized in 2 ml of saline supplemented with 60 μg each of amikacin and piperacillin per ml. Serial 10-fold dilutions were made, plated on blood agar supplemented with 1% culture filtrate for *H. capsulatum* or Sabouraud dextrose agar for *C. immitis*, and incubated at 37 or 25°C, respectively. Colonies were enumerated and data were calculated, with results expressed as CFU per gram of tissue.

Histopathology. Tissue specimens from spleens, lungs, and livers of nude mice (three mice per group) on day 20 after transfer and infection or instillation of saline were fixed in 10% buffered Formalin, embedded in paraffin, sectioned every 5 mm, and stained with hematoxylin and eosin, periodic acid-Schiff stain, and methenamine-silver (Grocott).

Statistical analyses. Differences between groups were compared by Student's *t* test, and significance was defined as $P < 0.05$. Mortality data were analyzed by Mantel-Haenszel survival analysis or Wilcoxon analysis, and $P < 0.05$ was considered significant.

RESULTS

Recovery of *H. capsulatum* from target organs. In the first set of experiments, clones 2.3H3 and 1S6 were transferred to C57BL/10 nude or irradiated (5 Gy) *nu*/+ mice. Two hours after transfer, the animals were infected intranasally with 5×10^6 *Histoplasma* yeasts. On day 5 of infection, the lungs, livers, and spleens were removed, and the number of CFU per gram of tissue was quantitated. Transfer of 2.3H3 significantly ($P < 0.05$) reduced the recovery of *H. capsulatum* yeasts from all organs of nude and *nu*/+ mice compared with recovery of yeasts from organs of mice that received 1S6 (Table 1).

TABLE 1. Effect of 2.3H3 and 1S6 cells in nude and *nu*/+ mice on clearance of *H. capsulatum* in lung, liver, and spleen tissue on day 5 of infection[a]

Mice	Organ	CFU/g of tissue (mean ± SD)[b]	
		2.3H3	1S6
Nude	Lung	$(6.5 \pm 4.2) \times 10^5$	$(38.3 \pm 2.5) \times 10^5$
	Liver	$(8.7 \pm 5.2) \times 10^3$	$(63.8 \pm 2.9) \times 10^3$
	Spleen	$(8.9 \pm 4.9) \times 10^3$	$(96.2 \pm 5.2) \times 10^3$
nu/+	Lung	$(12.2 \pm 9.2) \times 10^5$	$(271.4 \pm 242.4) \times 10^5$
	Liver	$(5.7 \pm 4) \times 10^3$	$(105 \pm 118) \times 10^3$
	Spleen	$(24.5 \pm 17) \times 10^3$	$(210 \pm 250) \times 10^3$

[a] Groups of six nude and nine *nu*/+ irradiated (5 Gy) C57BL/10 mice were inoculated with 10^7 resting T cells and then infected intranasally with 5×10^6 *H. capsulatum* cells. The mice were sacrificed on day 5 postinfection.
[b] Differences between 2.3H3 and 1S6 were significant ($P < 0.05$) for each type of mouse and organ (*t* test). Results of one representative experiment of three (nude) or four (*nu*/+) are shown.

Subsequently, we asked whether the protective effect of 2.3H3 would persist beyond day 5. Irradiated *nu*/+ mice were inoculated with 10^7 2.3H3 or 1S6 cells. Two hours later, the mice were infected intranasally with 5×10^6 *Histoplasma* yeasts, and on day 10 of infection, the mice were sacrificed. The lungs, livers, and spleens of mice given 2.3H3 contained fewer *H. capsulatum* CFU than the organs of animals that received 1S6 (Table 2). However, these differences were not statistically significant.

Since it appeared that the efficacy of 2.3H3 was transient, additional experiments were performed to determine whether a second injection of T cells on day 5 would enhance clearance of *H. capsulatum* on day 10. Nude (eight per group) or irradiated *nu*/+ (nine per group) mice were inoculated i.v. with 2.3H3 or 1S6 cells on day 0. Two hours later, they received 5×10^6 yeast cells intranasally. On day 5, the animals were again inoculated i.v. with 10^7 T cells. The mice were sacrificed on day 10, and the numbers of CFU in their livers, lungs, and spleens were determined. Despite an additional injection of T cells, there was no statistical difference ($P > 0.05$) in the numbers of CFU in organs between animals given 2.3H3 and those given 1S6. For example, the lungs of mice given two injections of 2.3H3

TABLE 2. Effect of 2.3H3 and 1S6 cells in *nu*/+ mice on clearance of *H. capsulatum* in lung, liver, and spleen tissue on day 10 of infection[a]

Organ	CFU/g of tissue (mean ± SD)[b]	
	2.3H3	1S6
Lung	$(12.7 \pm 12.8) \times 10^7$	$(18.7 \pm 18.7) \times 10^7$
Liver	$(0.54 \pm 0.58) \times 10^7$	$(2.4 \pm 2.8) \times 10^7$
Spleen	$(8.64 \pm 8.32) \times 10^7$	$(20.7 \pm 23.9) \times 10^7$

[a] Groups of seven to nine *nu*/+ irradiated (5 Gy) C57BL/6 mice were inoculated with 10^7 resting T cells and infected intranasally with 5×10^6 *H. capsulatum* cells. The mice were sacrificed on day 10 postinfection.
[b] Differences between 2.3H3 and 1SC were not significant ($P > 0.05$) for any of the three organs (*t* test). Results of one representative experiment of three are shown.

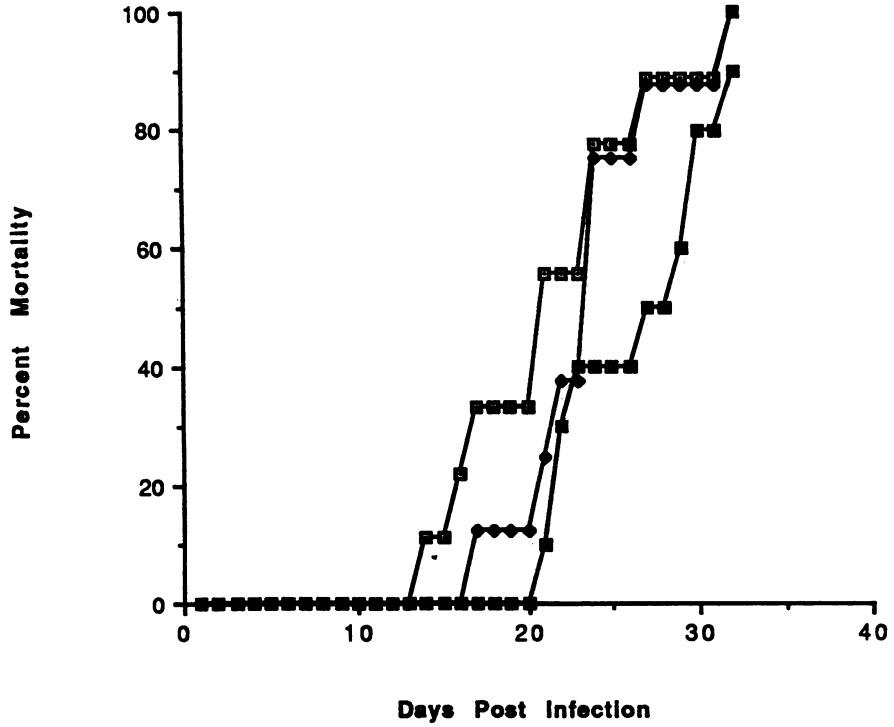


FIG. 1. Survival of mice given an injection of a T-cell clone. Groups of 8 to 10 nude C57BL/10 mice were given injections of 10^7 2.3H3 or 1S6 T cells or RPMI 1640 and then infected with 5×10^7 *H. capsulatum* cells intranasally. The *P* values obtained by comparing each pair of groups by Mantel-Haenszel survival analysis were >0.05. Results from one representative experiment of two are shown. Symbols: ⊡, 1S6; ◆, RPMI 1640; ■, 2.3H3.

contained $(9.8 \pm 13.3) \times 10^7$ CFU, whereas the lungs of animals that received 1S6 had $(15.2 \pm 17.4) \times 10^7$ CFU.

Survival of mice given T-cell clones. Nude mice were given an injection of 10^7 2.3H3 or 1S6 cells or RPMI 1640, and 2 h later, the mice were inoculated with 5×10^7 yeast cells intranasally. The mice were observed daily for mortality. No differences in survival were noted among the three groups (Fig. 1). Taken together, the data indicate that transfer of the T-cell clone 2.3H3 could transiently reduce the number of CFU but could not reduce mortality in mice given a lethal inoculum ($P > 0.05$; Mantel-Haenszel survival analysis comparing all animal groups).

In vivo specificity of 2.3H3. Irradiated C57BL/10 *nu*/+ mice were given 10^7 2.3H3 or 1S6 cells i.v. and 2 h later were infected intranasally with 40 arthroconidia of *C. immitis*. On day 5 of infection, the lungs, liver, and spleen were removed and cultured for *C. immitis*. The lungs of mice that received 2.3H3 contained numbers $[(5.2 \pm 4.5) \times 10^4]$ of CFU similar to those of mice given 1S6 $[(4.4 \pm 2.7) \times 10^4$ CFU] ($P > 0.05$). No CFU were detectable in livers or spleens of either group of animals. This finding strongly suggests that the in vivo activity of 2.3H3 is specific for *H. capsulatum*.

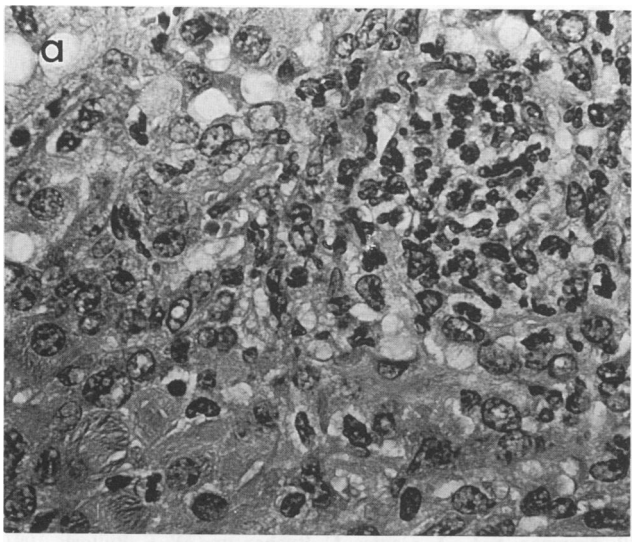
Histopathologic examination of organs. Lung sections of nude mice infected for 20 days and given 2.3H3 revealed the presence of granulomatous inflammation. Rare clusters of neutrophils were evident in alveoli and the peribronchiolar region. Fibrin deposition was noted in the alveoli and bronchioles (Fig. 2a). Granulomatous inflammation also was present in infected mice given 1S6. The lungs of these animals contained an exudative neutrophilic infiltrate, and fibrin was observed in alveolar macrophages and peribronchiolar macrophages. Quantitation of yeast cells per high-power field demonstrated that the lungs of mice injected with 2.3H3 contained fewer (5.85 ± 4.73) yeast cells per high-power field than the lungs of mice given 1S6 $(42.8 \pm 53.8$ yeast cells per high-power field) (Fig. 2b and c). However, no statistical difference was reached ($P > 0.05$).

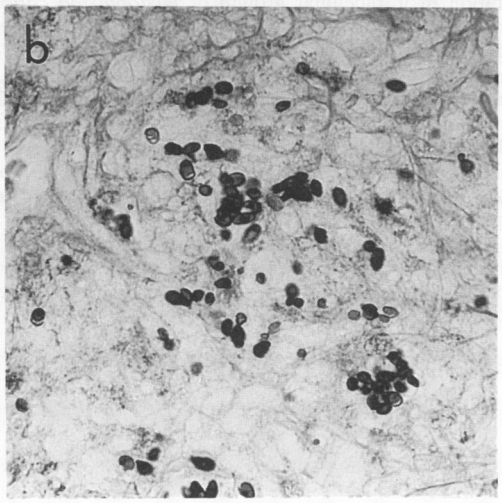
Granulomatous infiltration of livers of nude mice given 2.3H3 was located predominantly in the portal and perivenular areas. Numerous lobular aggregates of primarily mononuclear inflammatory cells were present, as well as multinucleated giant cells. Focal collections of neutrophils and coagulative necrosis were present (Fig. 3). The findings for livers of mice that were given 1S6 were similar.

In spleens of mice receiving either 2.3H3 or 1S6, there was extensive granulomatous inflammation involving but not confined to the red pulp. Again, the spleens of mice that received 2.3H3 contained fewer yeast cells per high-power field (1.6 ± 1.67) than those of mice that were given 1S6 (44.3 ± 56.9), although again, no statistically significant difference was reached ($P > 0.05$).

DISCUSSION

In experimental models and in the human disease condition, $CD4^+$ T cells are a critical constituent of host defenses against *H. capsulatum* (4, 6). Since T cells are organized clonally, it has been our goal to analyze the functional activity of monoclonal rather than polyclonal populations of $CD4^+$ T cells. The importance of studies of monoclonal T cells is that they provide insights into the immunological activity of a discrete and highly homogeneous population of cells. However, the utility of cloned T cells has, at times, been limited.





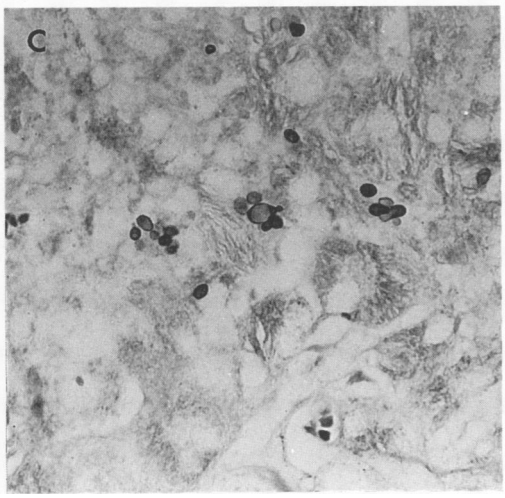


FIG. 2. (a) Section of a lung from a nude C57BL/10 mouse which was given an injection of 10^7 2.3H3 cells, displaying a mixed neutrophilic exudative and granulomatous inflammatory cell infiltrate. The section was stained with hematoxylin and eosin. Magnification, ×400. (b) Grocott methenamine-silver-stained section of a lung from a nude C57BL/10 mouse which received 10^7 1S6 cells, demonstrating numerous organisms. Magnification, ×400. (c) Grocott methenamine-silver-stained section of a lung from a nude B10 mouse which received 10^7 2.3H3 cells, demonstrating a lower burden of organisms. Magnification, ×400.

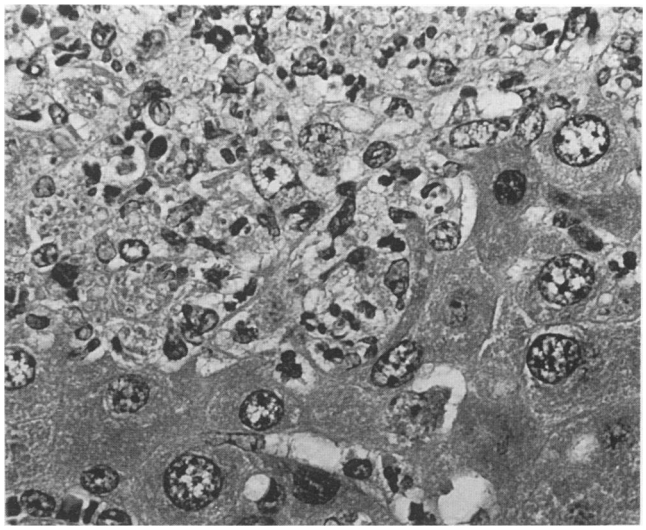


FIG. 3. Section of liver from a nude C57BL/10 mouse which was given 10^7 1S6 cells, demonstrating a granulomatous infiltrate. Numerous organisms are present within macrophages. The section was stained with hematoxylin and eosin. Magnification, ×400.

An earlier report indicated that supernatants from oligoclonal or monoclonal *H. capsulatum*-reactive T cells that were stimulated with antigen could inhibit the in vitro growth of intracellular yeasts in peritoneal macrophages; thus, these T cells did exert a protective function in an in vitro system (3). However, when these cells were transferred i.v. into normal, intact mice, they failed to express a protective effect. One explanation for this finding was that these cells did not traffic to the lymphoid organs (2).

In contrast, the results of the present study indicated that i.v. transfer of an *H. capsulatum*-reactive murine T-cell clone into nude or irradiated *nu*/+ mice can provide transient protection against an intranasal challenge with yeast cells. Moreover, the effect of this clone was specific for *H. capsulatum*, since it failed to alter infection with the heterologous fungal pathogen *C. immitis*. Thus, nude mice and irradiated mice are permissive hosts with which to study the in vivo potential of T-cell clones to confer protection.

Why clone 2.3H3 mediated protection in nude or irradiated mice but not in normal mice is not completely understood. One possibility is that nude mice or irradiated mice lack a regulatory cell population that alters the homing of T cells to sites of infection. If that is the case, then that cell population is also cyclophosphamide resistant, since injection of monoclonal T cells into normal mice treated with this alkylating agent did not exert protective function (2).

It is noteworthy that the effect of 2.3H3 was transient. Injection of this clone concomitant with infection did not sterilize host tissues. Indeed, by day 10 of infection, there was no statistical difference in organ CFU between groups of animals given 2.3H3 and groups given 1S6. Additional evidence that the protective capacity of 2.3H3 was limited

was that it failed to enhance survival in mice challenged with a lethal inoculum. Attempts to supplement the action of 2.3H3 by another injection of T cells at day 5 did not produce any measurable enhancement of protection.

It should not be surprising that a single clone cannot completely eliminate infection with *H. capsulatum*. T-cell clones respond to a highly restricted repertoire of peptide epitopes (7). Maximal activation of T cells requires that the epitope(s) be expressed in association with major histocompatibility complex antigens in optimal amounts. Too little epitope may not affect activation, and excess epitope may lead to peripheral anergy (1, 8). Therefore, the ability of the clone to release cytokines involved in antifungal activity may be critically dependent on the quantity of an epitope or epitopes on antigen-presenting cells. Any deviation from the optimal range may cause a failure to activate T cells.

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