INFECTION AND IMMUNITY, Nov. 1989, p. 3581–3587
0019-9567/89/113581-07$02.00/0

Vol. 57, No. 11

Role of L3T4$^+$ Lymphocytes in Protective Immunity to Systemic *Candida albicans* Infection in Mice

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Received 31 March 1989/Accepted 4 August 1989

Protective immunity to lethal *Candida albicans* challenge in vivo and activation of splenic macrophages with highly candidacidal activity in vitro were detected in mice infected with low-virulence agerminative yeast cells of the variant strain PCA-2, at a time when a strong delayed-type hypersensitivity (DTH) reaction to *C. albicans* occurred in the footpads of PCA-2-treated mice. The DTH reaction was transferable with spleen cell populations from these animals, and enrichment of splenic lymphocytes in L3T4$^+$ cells significantly increased the footpad swelling. The reactivity transferred by L3T4$^+$ cells was a radiosensitive (2,500 rads in vitro) phenomenon that required collaboration with radioresistant, silica-sensitive syngeneic cells in the host and was inhibited by treatment of recipient mice with antibodies to the L3T4 antigen or murine gamma interferon. In vitro, the PCA-2-immune L3T4$^+$ cells produced various lymphokine activities upon incubation with *C. albicans*, including gamma interferon and granulocyte-macrophage colony-stimulating factor. Anti-L3T4 monoclonal antibody treatment of PCA-2-infected mice significantly impaired their footpad reaction and resistance to *C. albicans*, as shown by increased recovery of yeast cells from the kidneys of anti-L3T4-treated mice. These results suggested that the mechanisms of anti-*Candida* resistance induced by PCA-2 may involve specific induction of a DTH response mediated by inflammatory L3T4$^+$ T cells and lymphokine-activated phagocytic effectors. However, the survival rate of the PCA-2-immune mice challenged with *C. albicans* was not significantly modified by administration of the anti-L3T4 antibody, thus allowing for the conclusion that compensatory mechanisms lead to considerable anti-*Candida* resistance when the activity of L3T4$^+$ cells is deficient.

Infection with *Candida albicans* induces both humoral and cell-mediated immune responses against the yeast (38). Anti-*Candida* antibodies confer only partial protection against experimental challenge, and cell-mediated immunity is thought to be a major factor in host resistance (33, 40). Although the importance of T-cell-dependent immunity in chronic mucocutaneous candidosis has been clearly established (12, 29, 34, 50), the protective role of this component of immunity in systemic candidosis has not been so well defined (13, 23, 46). As a matter of fact, innate natural immune mechanisms appear to be most important in resistance to disseminated *C. albicans* infections, with a major role apparently being played by nonspecifically activated polymorphonuclear leukocytes (PMN) (2, 6, 23, 33, 47) and macrophages (1, 3, 5, 30, 43).

The L3T4$^+$ T-cell subset actively participates in the regulation of immunological and inflammatory processes involving the functions of professional phagocytes (24). In experimental models of microbial infection, helper L3T4$^+$ (or T_H2) cells (11) appear to be essential for effective humoral immune responses to extracellular pathogens, while inflammatory L3T4$^+$ T (or T_H1) cells are responsible for T-cell-dependent macrophage activation and elimination of intracellular pathogens (9). In most of these infection models, a common feature of host-protective inflammatory L3T4$^+$ lymphocytes is their ability to release lymphokines that result in macrophage activation and development of a delayed-type hypersensitivity (DTH) reaction to microbial antigens (14, 21, 26, 28, 37, 41, 42).

In a previously described model of systemic infection of mice with *C. albicans*, we have shown that protective immunity to lethal challenge is induced by vaccinating the animals with low-virulence *Candida* cells of the agerminative variant strain PCA-2 (7). In this model, the establishment of effective protection requires a period of 7 to 14 days after vaccination, is accompanied by a state of chronic infection involving a critical antigenic load (51), and is apparently mediated by nonspecifically activated professional phagocytes with microbicidal properties (8). Because of the reported occurrence of DTH reactions to *Candida* antigens (23), in the present work we addressed the question of whether L3T4$^+$ T-cell-dependent immunity could be detected in mice infected with PCA-2 and whether this event might participate in the anti-*Candida* resistance induced by vaccination. Our results suggest an important role for inflammatory L3T4$^+$ cells as a cofactor of phagocytic cell activation, leading to an optimally protective response to *C. albicans* infection; however, they also suggest that compensatory mechanisms may provide substantial protection under conditions for which the activity of this T-cell subset is reduced.

MATERIALS AND METHODS

Mice. Hybrid (BALB/cCr × DBA/2Cr)F_1 (CD2F1; *H-2^d/H-2^d*) and inbred C57BL/6 (*H-2^b*) mice were obtained from Charles River Breeding Laboratories, Calco, Milan, Italy. Both male and female mice ranging in age from 2 to 4 months were used.

Yeasts. The origin and characteristics of the two strains of *C. albicans* (CA-6 and PCA-2) used in this study have already been described in detail (7, 8, 51). Briefly, the highly pathogenic strain CA-6 was isolated from a clinical specimen; the agerminative, low-virulence strain PCA-2 is an echinocandin-resistant mutant of the parent strain 3153A and

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was kindly supplied by D. Kerridge, Department of Biochemistry, University of Cambridge, Cambridge, England. All yeasts were grown to the stationary phase at 28°C under slight agitation in low-glucose Winge medium composed of 0.2% (wt/vol) glucose and 0.3% (wt/vol) yeast extract (BBL Microbiology Systems, Cockeysville, Md.). Under these conditions, cultures gave a yield of 2×10^8 to 4×10^8 cells per ml, and the organisms grew as an essentially pure yeast-phase population. After the 24-h culture, cells were harvested by low-speed centrifugation ($1,000 \times g$), washed twice in saline, and diluted to the desired density.

DTH assay. A direct assay system for measuring the DTH response to cell surface antigens was used (44), in which heat-inactivated CA-6 cells (2×10^6 cells per 0.04 ml of pyrogen-free saline) were inoculated into the footpads of mice that had been infected intravenously (i.v.) with 10^6 live PCA-2 cells a number of days before. In the DTH transfer experiments, CA-6 cells were injected into the footpads of mice that had been adoptively transferred, 24 h earlier, by i.v. administration of lymphocytes (10^7 lymphocytes per 0.5 ml of RPMI 1640 medium) from PCA-2-infected hosts. The DTH reaction was recorded 24 h later by weighing the footpad as a measure of swelling, and the results were expressed as the increase in right hind footpad weight over that of the saline-injected left hind counterpart. Data are the means ± standard errors for six to eight mice per group.

Positive selection of L3T4$^+$ cells. Positive selection of L3T4$^+$ cells was done as previously described (32, 44). Briefly, B-cell-depeleted splenic lymphocytes from mice infected with PCA-2 14 days earlier (unless otherwise stated) were incubated with rat monoclonal anti-mouse L3T4 immunoglobulin G (IgG) antibody (GK 1.5; a generous gift from Frank Fitch, University of Chicago, Chicago, Ill.) for 40 min at 4°C. The cells were washed, resuspended, and placed in petri dishes coated with a goat anti-mouse IgG antiserum cross-reacting with rat antibody determinants (32) (a gift from M. Mage, National Institutes of Health, Bethesda, Md.). The nonadherent cells (>90% negative for L3T4 antigen on immunofluorescence analysis) were removed, and the plates were washed four times with phosphate-buffered saline. The attached cells were recovered by vigorous pipetting of Hanks balanced salt solution onto the cell layer, centrifuged, and suspended in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum. These cells (>98% positive for L3T4 antigen) were referred to as L3T4$^+$ cells.

In vivo treatments with antibodies and silica. For assessment of the in vivo effects of monoclonal antibodies (MAbs) on the footpad reaction to *C. albicans*, mice were injected i.v. with 0.5 ml of GK 1.5 or R4-6A2 (ATCC HB 170) hybridoma culture supernatants by methods previously shown to affect the induction and expression of antigen-specific DTH responses (44). Supernatants, which were taken at the time of medium exhaustion, centrifuged, and filtered, typically contained 8 to 10 μg of rat IgG antibody per ml, as measured in an enzyme-linked immunosorbent assay with an affinity-purified rat IgG antibody as a reference preparation of known titer (0.5 mg/ml). Silica (Santocel; Monsanto, St. Louis, Mo.) was dissolved in saline (5 mg/ml), heated in boiling water, and administered intraperitoneally to mice (3 mg per mouse) 24 h before adoptive transfer of lymphocytes. In a study of the effect of anti-L3T4 treatment on the in vivo resistance of mice to CA-6 challenge, the animals each received five injections of GK 1.5 hybridoma culture supernatant, as illustrated above.

Plate counts. Pooled spleen cells (5×10^5 cells in 0.1 ml per well) from three to five mice were infected with CA-6 cells (5×10^4 cells in 0.1 ml of suspension per well). After a 4-h incubation at 37°C in 5% CO_2, the plates were vigorously shaken and serial dilutions from each well were made in distilled water. Pour plates (quadruplicate samples) were made by spreading each sample on Sabouraud dextrose agar. The number of CFU was determined after 18 h of incubation at 37°C. Control cultures consisted of *C. albicans* incubated without effector cells.

Titration of CA-6 in the kidneys. At various times after challenge, the mice were killed by cervical dislocation, and the kidneys from individual mice were aseptically removed and placed in a tissue homogenizer with 6 ml of sterile saline. The number of CFU of CA-6 in the specimens (five mice per time point) was determined by a plate dilution method in which differential counts between CA-6 and PCA-2 were based on morphological examination of the colonies on chlamydospore agar (Biolife Italiana, Milan, Italy). The colonies were counted after 48 to 72 h of incubation at 37°C, and results (means ± standard errors) were expressed as the number of CFU per organ.

Production of culture supernatants containing lymphokine activity. Supernatants from mixed lymphocyte-*Candida* cultures were obtained as follows. Splenic lymphocytes (2×10^7) suspended in RPMI 1640 medium supplemented with 0.5 fetal calf serum and 5×10^{-5} M 2-mercaptoethanol were cultured with heat-inactivated CA-6 cells at a lymphocyte-to-yeast cell ratio of 80:1 in a volume of 1 ml in 16-mm plastic culture wells. On assaying positively selected L3T4$^+$ cells, 10% plastic-adherent macrophages from intact mice were added to the cultures. After incubation at 37°C in a CO_2 incubator for 20 h, culture supernatants were harvested by centrifugation and assayed for lymphokine activity.

Assays for lymphokine activity. Interleukin-2 (IL-2) activity was measured by its ability to sustain the growth of an IL-2-dependent line, HT-2 (25), by using the tetrazolium-based colorimetric assay (35). This assay, also used to assess the effect of other lymphokines on cell growth and survival, involves treating cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37°C for 4 h, washing them with phosphate-buffered saline, solubilizing formazan crystals with acidified isopropanol, and reading the A_{540} with a microELISA reader. IL-2 titers were expressed as units per milliliter, which were calculated by comparing the dilutions of the test supernatant that gave 50% maximal stimulation of HT-2 proliferation with a standard preparation of IL-2 (1.5×10^6 U/ml; Cetus Corp., Emeryville, Calif.).

Culture supernatants were analyzed for lymphotoxin (LT) activity as described previously (19). Briefly, samples were serially diluted and added to mouse L929 cells. At 72 h later, cell survival was assessed and the percent cytotoxicity for each dilution was calculated. LT titers were expressed as units per milliliter, obtained by referring to the activity of a human recombinant LT preparation (1.2×10^8 U/ml; a gift of M. Mage).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) activity was determined by its ability to sustain the growth of bone marrow cells in soft agar cultures (48). Bone marrow cells (10^5 in 1 ml) were plated in 35-mm petri dishes, and 0.1-ml volumes of serial dilutions of the test supernatants were added. Granulocyte-macrophage CFU (>50 cells) were examined at ×40 magnification. GM-CSF titers were expressed as units per milliliter by referring to the standard curve obtained with a recombinant GM-CSF preparation (2×10^3 U/ml; Genzyme Corp., Boston, Mass.).

Interferon (IFN) activity was measured in terms of the

protection of mouse L929 cells from the cytopathic effect of vesicular stomatitis virus as described by Palmer et al. (39). Briefly, 0.1-ml of twofold dilutions of test supernatant and 5 × 10^4 L929 cells in 0.1 ml were incubated in flat-bottom microdilution plates for 18 to 24 at 37°C in a 5% CO_2 incubator. Cultures were then drained, and the monolayers in each well were challenged with 50 μl of vesicular stomatitis virus (5 × 10^6 PFU/ml), resulting in complete cytopathic effect of unprotected cells within 48 h. IFN titers were expressed as units per milliliter, as estimated by reference to the standard curve established with a recombinant IFN-γ preparation (1 × 10^4 U/ml; Genentech Inc., South San Francisco, Calif.). In selected experiments, test supernatants to be assayed for antiviral activity were admixed with anti-IFN-γ monoclonal antibody (R4-6A2 hybridoma culture supernatant; final dilutions, 1/200 to 1/4,000) before being incubated with L929 cells. This resulted, as a rule, in complete neutralization of the antiviral activity with antibody dilutions up to 1/2,000.

Irradiation. Mice were exposed to 800 rads of total-body irradiation before lymphocyte transfer, in a ^{60}Co irradiator delivering gamma rays at 900 rads/min. The same ^{60}Co bomb was used for irradiation of L3T4+ cells in vitro, at room temperature, in sealed glass vials.

Statistical analysis. Differences in survival times were determined by the Mann-Whitney U test. Differences in the numbers of CFU in the kidneys were analyzed by Student's *t* test. The same test was performed on footpad weight increase values in the DTH experiments. Each experiment was performed at least three times.

RESULTS

Effect of PCA-2 injection on anti-*Candida* resistance, candidacidal activity of spleen cells, and delayed-type footpad reaction to *Candida* antigens. CD2F1 mice inoculated with live PCA-2 cells at different times were either challenged i.v. with 10^6 CA-6 cells or injected in the footpad with inactivated *Candida* cells to be assayed 24 h later for footpad swelling, as described in Materials and Methods. Additional groups of mice treated with PCA-2 served as donors of spleen cells to be reacted in vitro with *Candida* cells in a CFU inhibition assay. In line with our previous results (7), PCA-2 treatment conferred considerable protection against microbial challenge performed 10 to 14 days after vaccination, at a time when highly candidacidal effectors were found in the spleens of PCA-2-infected mice (Table 1). Concurrent with maximal anti-*Candida* resistance was the detection of strong reactivity to *Candida* antigens in the footpads of sensitized mice. Histopathological evaluation of footpads indicated that the cellular response was predominantly mononuclear. Neither protective immunity nor DTH reactivity was detected in mice sensitized 14 days earlier with heat-inactivated CA-6 cells.

Transfer of DTH with spleen cells and L3T4+ lymphocytes. In mice, the inflammatory response known as DTH is mediated by T cells with the L3T4+ surface antigen phenotype. The possible involvement of L3T4+ lymphocytes in mediating DTH to *C. albicans* was investigated in experiments of adoptive transfer of DTH. By using the GK 1.5 MAb to the L3T4 antigen, we performed the nonlytic separation of L3T4+ and L3T4− cells present in the splenic populations of mice infected with PCA-2 2, 6, 14, 22, 42, or 60 days earlier. The positively selected L3T4+ lymphocytes (10^7) were then injected i.v. into recipient mice, whose right hind footpads were inoculated 24 h later with heat-inactivated *Candida* cells. After an additional 24 h, the DTH response was measured as described above. Unfractionated splenic populations from mice given PCA-2 14 days earlier or from intact mice were also assayed. A DTH reaction to *Candida* antigens occurred following adoptive transfer of splenocytes from mice treated with PCA-2 14 days earlier, and the response was enhanced by the use of positively selected L3T4+ cells (Fig. 1). Moreover, the kinetics of emergence of DTH reactivity transferred by L3T4+ cells closely mirrored the development of protective immunity to *Candida* challenge in the PCA-2-treated hosts (Table 1) (7).

TABLE 1. Effect of PCA-2 treatment on mouse resistance to *C. albicans* challenge

Day of treatment[a]	Results of CA-6 challenge[b]		Candidacidal activity[c]	Footpad wt increase (mg)[d]
	MST	No. dead/ total no.		
Untreated control	3	10/10	30.7 ± 2.0	2.2 ± 0.91
3	5	10/10	38.0 ± 3.2	4.9 ± 0.87
10	14[e]	6/10	66.8 ± 5.1[e]	7.3 ± 1.22[e]
14	>60[e]	0/10	68.2 ± 4.3[e]	11.8 ± 1.73[e]
60	4	10/10	35.2 ± 3.1	3.9 ± 0.87
14[f]	3.5	10/10	33.8 ± 2.0	3.2 ± 0.65

[a] PCA-2 (10^6 cells per mouse) was given as a single i.v. injection a number of days before systemic challenge with CA-6, or footpad injection of inactivated CA-6 cells in a DTH assay, or collection of spleen cells to be tested for CFU inhibitory activity.

[b] 10^6 CA-6 cells per mouse were injected i.v. MST, Median survival time; No. dead/total no., number of dead mice at 60 days/total number of animals tested.

[c] Percent CFU inhibition. Results are the means ± standard error at an effector-to-target (CA-6) cell ratio of 10:1.

[d] The increase in footpad weight as measured 24 h after footpad injection of heat-inactivated CA-6 cells.

[e] $P < 0.01$ (PCA-2-treated mice versus controls).

[f] Heat-inactivated CA-6 cells (10^6 cells per mouse) were injected i.v. in place of PCA-2 cells 14 days before challenge.

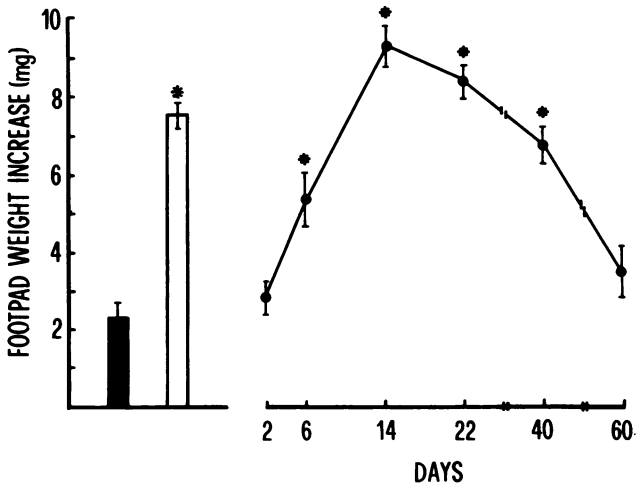

FIG. 1. Footpad reaction to CA-6 challenge in mice adoptively transferred with PCA-2-immune lymphocytes. Positively selected L3T4+ cells from mice given PCA-2 a number of days earlier (●) were injected i.v. into recipient hosts to be assayed 24 h later for DTH to *Candida* antigen. Unfractionated splenocytes from mice given PCA-2 14 days earlier (□) and nonimmune spleen cells (■) were also assayed. Symbol: *, $P < 0.01$ (PCA-2-treated mice versus nonimmune controls).

TABLE 2. Effect of host treatment on footpad reaction to CA-6 cells

Type of lymphocyte[a]	In vivo treatment[b]	Mean footpad wt increase ± SE (mg)
Expt 1		
NSC	None	2.8 ± 0.54
L3T4$^+$	None	8.2 ± 1.59[c]
L3T4$^+$	Medium	8.6 ± 0.41[c]
L3T4$^+$	GK 1.5 MAb	2.5 ± 0.55
Expt 2		
NSC	None	2.7 ± 0.60
L3T4$^+$	None	9.1 ± 0.97[c]
L3T4$^+$	R4-6A2 MAb	3.6 ± 0.96
L3T4$^+$	Silica	2.3 ± 0.82

[a] Non-immune spleen cells (NSC) or PCA-2-immune L3T4$^+$ lymphocytes (10^7) were injected i.v. into mice to be challenged by footpad injection of CA-6 cells.

[b] Animals to be assayed for footpad reaction received injections of hybridoma culture supernatants or silica particles by different procedures (see text for details).

[c] $P < 0.01$ (significantly higher than in the nonimmune spleen cell group).

Inhibition of DTH transfer by MAbs and silica. To gain some insight into the mechanisms of the anti-*Candida* DTH response conferred by L3T4$^+$ cells, we resorted to in vivo selective depletion of immune functions in hosts injected with these cells and subsequently assayed for DTH reactivity to *C. albicans*. Mice were injected with 0.5 ml of GK 1.5 hybridoma culture supernatant on the day before and 1 h after the adoptive transfer of PCA-2-immune L3T4$^+$ cells, before CA-6 footpad challenge and measurement of the DTH response. The GK 1.5 antibody completely inhibited the development of a footpad reaction (Table 2, experiment 1). To study the possible involvement of IFN-γ and macrophages in our DTH model, we treated the prospective recipients of the footpad challenge either with antibodies to IFN-γ or with silica particles, a proven antimacrophage agent (44). Because rat R4-6A2 IgG MAb to murine IFN-γ blocks lymphokine-induced macrophage activation (49), culture supernatants of the corresponding hybridoma cell line were injected i.v. (0.5 ml per mouse) the day before and minutes after *Candida* footpad challenge in our DTH assay system. In addition, a group of mice that did not receive antibodies were treated with an intraperitoneal injection of silica particles (3 mg per mouse) before inoculation of CA-6, into the footpads; a procedure previously shown to decrease the resistance of PCA-2-treated mice to systemic *Candida* infection was used (8). The results of experiment 2 in Table 2 show that both the MAb and the antimacrophage agent inhibited the development of a footpad reaction to CA-6.

Transfer of DTH to irradiated and allogeneic hosts. The occurrence of a delayed-type footpad reaction to *C. albicans* in irradiated and allogeneic recipients of the PCA-2-immune lymphocytes was also investigated. For this purpose, positively selected L3T4$^+$ cells were injected i.v. into lethally irradiated (800 rads) CD2F1 or intact C57BL/6 hosts. Moreover, a group of intact CD2F1 mice received L3T4$^+$ cells exposed to 2,500 rads in vitro. L3T4$^-$ cells were also tested. The animals were then assayed for their ability to mount a DTH response to CA-6 cells. Lethal irradiation of the host before adoptive lymphocyte transfer had no effect on the DTH response (Table 3). In contrast, in vitro exposure of L3T4$^+$ cells to radiation greatly reduced their activity when they were transferred in vivo to intact recipients. No DTH activity was displayed by CD2F1 mouse lymphocytes when injected into *H-2*-incompatible recipients.

TABLE 3. DTH in allogeneic and irradiated recipients

Recipient mice	Transfer cells[a]	Mean footpad wt increase ± SE (mg)
CD2F1	Nonimmune cells	2.9 ± 0.71
CD2F1	L3T4$^+$ cells	10.9 ± 0.45[b]
CD2F1, 800 rads	L3T4$^+$ cells	8.8 ± 1.46[b]
CD2F1	Irradiated L3T4$^+$ cells	3.8 ± 0.77
CD2F1	L3T4$^-$ cells	4.1 ± 0.69
C57BL/6	L3T4$^+$ cells	3.8 ± 0.48

[a] Nonimmune spleen cells or PCA-2-immune L3T4$^+$ lymphocytes, either intact or irradiated (2,500 rads), were injected i.v. into mice to be challenged by footpad injection with CA-6 cells. L3T4$^-$ cells indicates the nonadherent cells (>90% negative for L3T4 antigen) resulting from the panning procedure.

[b] $P < 0.01$ (significantly higher than in the nonimmune spleen cell group).

Lymphokine secretion in vitro by PCA-2-immune L3T4$^+$ cells. Lymphokine production, which accounts for much of the T-cell activity, correlates with functional phenotype in L3T4$^+$ lymphocyte clones (24). Of these cells, the ones predominantly involved in DTH reactions have been termed inflammatory T or T_H1 (11) cells and are characterized by the release of a set of lymphokines (primarily IL-2, IFN-γ, LT, and GM-CSF) upon antigen stimulation in vitro. We therefore attempted to identify lymphokine activity in the supernatants from PCA-2-immune lymphocytes cultured in vitro with inactivated CA-6 cells. Unfractionated splenic lymphocytes or positively selected L3T4$^+$ cells mixed with syngeneic macrophages were cultured for 20 h with inactivated *Candida* cells before culture supernatants were tested for IL-2, LT, GM-CSF, and IFN-γ activities, as described in Materials and Methods. The results of a typical experiment are given in Table 4, which shows that all of these activities could be detected in supernatants from PCA-2-immune lymphocytes incubated with *C. albicans*. Minimal lymphokine activity was generated by L3T4$^+$ cells in the absence of either third-party macrophages (data not shown) or stimulator CA-6 cells (Table 4).

Effect of anti-L3T4 administration on resistance to *C. albicans*. We next investigated the effect of in vivo anti-L3T4 treatment on the protective immunity induced by PCA-2 to CA-6 challenge. Mice treated with PCA-2 on day −14 received five daily i.v. injections (days −1 to +3) of GK 1.5 hybridoma culture supernatant (a total of approximately 25 μg of anti-L3T4 MAb per mouse) by using a procedure that had been shown in preliminary experiments to reduce the number of L3T4$^+$ cells from approximately 30% to <1% in the spleens of the PCA-2-infected mice, as detected by immunofluorescence. On day 0, the animals were challenged with 5×10^5 CA-6 cells and then examined for mortality or the ability to clear *Candida* cells from their kidneys. Addi-

TABLE 4. Lymphokine production by PCA-2-immune lymphocytes

Source of supernatant[a]	Lymphokine activity (U/ml)[b]			
	IFN-γ	LT	IL-2	GM-CSF
Nonimmune spleen cells	0	7	0	12
PCA-2-immune cells, unfractionated	40	227	841	250
L3T4$^+$ cells	130	510	4,312	1,220
L3T4$^+$ without CA-6 cells	2	10	20	18

[a] Heat-inactivated CA-6 cells were cocultured with unfractionated spleen cells or a mixture of L3T4$^+$ and γ-irradiated (2,500 rads) splenic adherent cells for 20 h prior to collection of supernatants.

[b] Supernatants were tested for lymphokine activity as described in the text.

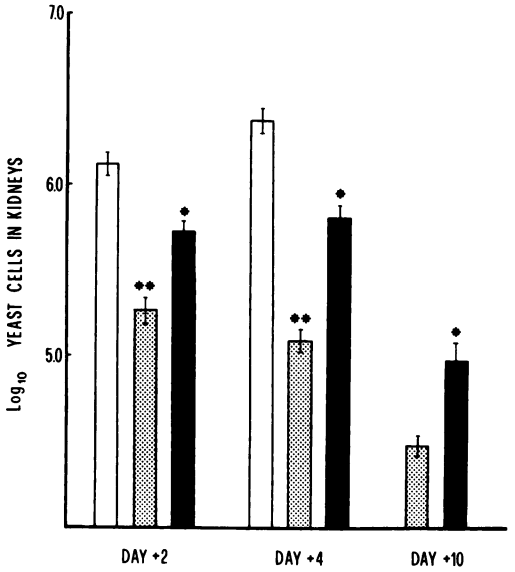

FIG. 2. Effects of anti-L3T4 treatment on the clearance of CA-6 cells from the kidneys. PCA-2-infected mice, treated with media (▨) or GK 1.5 hybridoma culture supernatant (■), were challenged i.v. on day 0 with 5×10^5 CA-6 cells. On days 2, 4, and 10, the CA-6 titer in the kidneys was measured. Symbols: □, control mice subjected to CA-6 challenge only (median survival time, 5 days); *, $P < 0.01$ (anti-L3T4-treated mice versus untreated controls); **, $P < 0.01$ (PCA-2-treated mice versus controls).

tional groups of mice, treated with PCA-2 and antibodies as described above but not subjected to microbial challenge, were either used as donors of splenocytes to be assayed in vitro for lymphokine activity or tested for DTH reactivity in vivo 24 h after the last anti-L3T4 antibody injection. MAb treatment significantly decreased the resistance of mice to *Candida* infection, in that more yeast cells were recovered from the kidneys of anti-L3T4-treated mice at days 2, 4, and 10 after infection (Fig. 2). However, the majority of these mice eventually cleared viable *Candida* cells from their organs, and the outcome of challenge was not significantly different from that for control mice receiving medium (Table 5). In addition, the anti-L3T4 treatment completely blocked the development of a DTH reaction to inactivated *Candida* cells (Table 5). Upon in vitro exposure to inactivated *Candida* cells as previously illustrated, unfractionated spleen cells from the anti-L3T4-treated mice produced no detectable IL-2 activity and as little as 20 U/ml of LT activity, in contrast to the strong production by control mice (IL-2, 905 U/ml; LT, 250 U/ml). The percentage of L3T4+ cells in the spleens of the antibody-treated mice remained very low for several days after treatment was discontinued, with repopulation beginning no earlier than 8 to 10 days after treatment was discontinued.

TABLE 5. Effect of anti-L3T4 treatment on mouse reactivity to *C. albicans* challenge

Treatment[a]	Results of CA-6 challenge[b]		Mean footpad wt increase ± SE (mg)
	MST	No. dead/ total no.	
None	5	10/10	2.6 ± 0.31
PCA-2	>60	0/10	8.4 ± 0.71[c]
PCA-2 + media	>60	0/10	9.1 ± 0.60[c]
PCA-2 + anti-L3T4 MAb[d]	>60	3/10[e]	3.2 ± 0.45

[a] PCA-2 (10^6 cells per mouse) was given as a single i.v. injection 14 days before systemic i.v. challenge with CA-6 (10^6 cells per mouse) or footpad injection of inactivated CA-6 cells in a DTH assay.
[b] MST, Median survival time; no. dead/total no., number of dead mice at 60 days/total number of animals tested.
[c] $P < 0.01$ (significantly higher than in the untreated control group).
[d] See text for details of treatment.
[e] Dead mice showed evidence of disseminated candidosis at autopsy.

DISCUSSION

There is increasing evidence showing that L3T4+ T lymphocytes are crucially involved in cell-mediated immune responses to intracellular pathogens (24). Although L3T4+ cells may also exert disease-promoting effects under special conditions (20, 31), the activity of T_H1 inflammatory lymphocytes appears to be host protective in most experimental infection models and is associated with the detection of DTH reactions to microbial antigens (10, 14, 26, 28, 41, 42, 45). Synthesis of IFN-γ and other lymphokines (namely, IL-2, LT, IL-3, and GM-CSF) is a distinctive feature of T_H1 inflammatory L3T4+ cells and is believed to play an important role in both induction of DTH and protective immunity to pathogens that inhabit macrophage vesicles (9, 21, 37). Recently, major histocompatibility complex class II-restricted cytolytic T cells with the L3T4+ surface phenotype have been described, and it has been suggested that these cells may participate in host resistance to intracellular pathogens (27, 36).

In *C. albicans* infections, mechanisms against the yeast are believed to involve specific T cells because patients with chronic mucocutaneous candidosis, malignancies, and immunodeficiency syndromes often display defective skin test DTH reactivity to *Candida* antigens and the T cells from a majority of these patients show no responsiveness to induction of proliferation by *Candida* antigen in vitro (38). In resistance to disseminated candidosis, the role of T-cell-dependent immunity is more controversial (13, 23, 46) and nonspecifically activated phagocytes are undoubtedly most important in combating the infection (2, 6, 40, 47). Because of the crucial involvement of T-cell-derived lymphokines in the regulation of phagocytic cell functions, it is possible that *Candida*-specific lymphocytes contribute to the activation of fungicidal effectors via the release of soluble mediators. In fact, DTH reactions are known to occur in response to *Candida* antigens (23); for this reason, in the present study we investigated the role of T inflammatory L3T4+ cells in resistance to systemic *Candida* infection in mice in which protective immunity had been induced by vaccination with low-virulence variant cells.

Our results indicated that the protection elicited by PCA-2 is indeed associated with the detection of DTH reactivity to CA-6 antigens, an effect transferable with L3T4+ lymphocytes from vaccinated mice. The kinetics of the emergence of this reactivity were so closely associated with those of emergence of protective immunity in the PCA-2-treated host that they suggested a causal relationship between the two phenomena. In agreement with previous results in other systems, the L3T4+ cells responsible for transfer of DTH appeared to be sensitive to radiation in vitro and to specific antibody in vivo, while they collaborated with radioresistant, silica-sensitive cells in the host (44). Conceivably, the requirement for host-derived macrophages in our model serves two purposes. Accessory functions must be provided to immune L3T4+ lymphocytes by syngeneic antigen-pre-

senting macrophages, as suggested by the failure of adoptively transferred L3T4$^+$ lymphocytes to induce a DTH response in allogeneic hosts in vivo; this might be reflected in vitro by the inability of positively selected L3T4$^+$ cells to produce lymphokines in response to CA-6 in the absence of plastic-adherent cells from naïve donors. On the other hand, host-derived macrophages might be among the effector cells activated by lymphokines produced by *Candida*-immune L3T4$^+$ cells.

The set of lymphokine activities produced in vitro by L3T4$^+$ cells in response to *C. albicans* included IL-2, LT, GM-CSF, and IFN-γ, all of which are known to be produced by T_H1 inflammatory lymphocytes. LT, GM-CSF, and IFN-γ all have profound effects on the functions of PMN and macrophagic cells. Increased GM-CSF production may account for the marked augmentation in the number of PMN and macrophages that occurs in the peripheral blood and spleens of mice injected with PCA-2, as previously reported (7, 8, 51). GM-CSF activates mature granulocytes (16) and cooperates with IFN-γ for induction of resistance to infection (4). IFN-γ, on the other hand, is a most potent macrophage activator (21, 49) and activates human PMN to candidacidal effectors (17). LT and tumor necrosis factor, a cytokine with substantial homology to LT, facilitate macrophage activation (22) and exert PMN-mediated candidacidal activity (17). These considerations, together with the results of previous studies in which a variety of pathogens were used (9, 10, 26, 28, 41, 42, 45), suggest that L3T4$^+$ cells provide a substantial contribution to host defense against infection with *C. albicans*.

To substantiate this hypothesis, we performed experiments involving selective depletion in vivo of L3T4$^+$ cells by using the specific MAb GK 1.5; this antibody, besides blocking the expression of adoptively transferred DTH reactivity to *C. albicans* as described above, has been extensively used in the past to study the in vivo role of L3T4$^+$ cells in a variety of experimental systems (for a review, see reference 14). Surprisingly enough, we found that although anti-L3T4 administration significantly impaired host resistance to *C. albicans*, DTH reactivity, and lymphokine production in vitro, most of the infected mice eventually cleared the yeast from their kidneys in a manner that was only delayed compared with that in non-antibody-treated control mice. Similar results were obtained by Czuprynski et al., who studied the effect of anti-L3T4 treatment on mouse resistance to *Listeria monocytogenes* infection (14). On the basis of the clearing rate of the listeriae from the organs of antibody-treated animals, these authors concluded that compensatory mechanisms allowed anti-L3T4-treated mice to eliminate the pathogen in a retarded but otherwise relatively normal fashion. Our present data are in agreement with such previous findings and also support the same conclusions. Undoubtedly, the nature of the compensatory mechanisms operating in our infection model remains to be determined. One possibility is that lymphokines with substantial activity on phagocytic cell functions are produced by L3T4$^-$ T (15) or non-T (18) lymphocytes when the activity of L3T4$^+$ cells is deficient. In this regard, it should be noted that in a previous study in which congenitally athymic mice (which are known to express high spontaneous levels of macrophage activation [13]) were used, PCA-2 treatment protected 50% of the vaccinated mice against CA-6 challenge (8).

In conclusion, our present data suggest that specifically immune L3T4$^+$ cells contribute to optimally protective immunity to systemic *Candida* infection through the intervention of soluble, possibly interactive mediators which cooperate to activate phagocytic cells with candidacidal activity. However, there is no absolute requirement for the lymphokines secreted by the PCA-2-immune T-inflammatory lymphocytes for the vaccine to be effective, since selective depletion of L3T4$^+$ cells only delays the elimination of yeast cells from the mouse organs. In addition, our data call attention to the notion that resistance to systemic candidosis does not necessarily correlate with expression of L3T4$^+$ cell-dependent activities, such as DTH (23) or lymphocyte activation in vitro in response to *Candida* antigen.

ACKNOWLEDGMENT

We thank Eileen Zannetti for her editorial assistance in the preparation of the manuscript.

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