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Th1 and Th2 Cytokine Secretion Patterns in Murine Candidiasis: Association of Th1 Responses with Acquired Resistance

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Two chemically mutagenized agerminative variants of *Candida albicans* were used to immunize mice against challenge with highly virulent cells of the parent strain. Although both mutants (Vir^- 3 and Vir^- 13) resulted in nonlethal infection and could be recovered from mouse organs for many days after the intravenous inoculation of 10^7 to 10^6 cells, significant protection to systemic challenge with virulent *C. albicans* was induced by only one (Vir^- 3) of the two variants. Anticandidal resistance in Vir^- 3-infected mice was associated with the occurrence in vivo of strong delayed-type hypersensitivity to *Candida* antigen, detection in vitro of highly fungicidal effector macrophages, and presence in the serum of a large proportion of *Candida*-reactive antibodies of the immunoglobulin G2a isotype. Bulk cultures of purified $CD4^+$ lymphocytes from mice infected with either mutant were compared for their ability to produce gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, and IL-6 in vitro. After stimulation with specific antigen, $CD4^+$ cells from Vir^- 3-immunized mice released large amounts of the Th1-specific cytokines, IFN-γ and IL-2, at a time when $CD4^+$ cells from Vir^- 13-infected mice predominantly secreted the characteristic Th2 cytokines, IL-4 and IL-6. These results were confirmed by quantitative analysis of cytokine-producing Th1 and Th2 cells. In addition, only mice infected with Vir^- 3 displayed a high frequency of $CD8^+$ cells with the potential for in vitro lysis of yeast-primed bone marrow macrophages. Purified $CD4^+$ cells from Vir^- 3-infected mice, but not a mixture of these cells with $CD4^+$ lymphocytes from mice infected with Vir^- 13, could adoptively transfer delayed-type hypersensitivity reactivity onto naive mice. Taken together, these data suggest that both Th1 and Th2 $CD4^+$ lymphocytes may be activated during experimental *C. albicans* infection in mice.

The outcome of host-parasite interactions in fungal infections is determined by the balance between the pathogenicity of the organism and the adequacy of the host defenses. A wide range of host defense mechanisms are involved in protection against *Candida albicans*; over the years, many studies have demonstrated roles for humoral factors, phagocytic cells, classical cell-mediated immunity, and antibody-producing cells (38), yet their relative contributions and the possible occurrence of regulatory mechanisms in resistance against specific forms of candidiasis in humans remain to be elucidated (1, 37, 41). Experimental models used to study invasive forms of candidiasis have generally exploited animals with no underlying immunity to the yeast and have confirmed the clinical finding that reduced neutrophil number and function, combined with the breakdown of anatomical defenses, are the critical factors predisposing immunocompromised or severely debilitated patients to invasive candidiasis (6). Experimental models have also been developed to study the mechanisms of acquired systemic anticandidal resistance under conditions more similar to those found in humans, in whom underlying immunity to the yeast is usually present. Long-term colonization of adult mice in the absence of immunosuppressive and antimicrobial treatment has been achieved by intragastric colonization of infant (15) or germfree (8) mice, or by systemic infection with agerminative yeast cells of a variant strain (5, 49). These studies have provided evidence that both specific cell-mediated immunity and phagocytic cell activity are instrumental in the optimal expression of acquired resistance to systemic candidiasis in mice. In particular, in the persistent infection of mice with attenuated yeast-phase *C. albicans*, it has been possible to demonstrate that development of systemic resistance to an otherwise lethal yeast challenge correlates with the emergence of a strong delayed-type footpad reaction to the yeast and with the detection of gamma interferon (IFN-γ)-secreting $CD4^+$ lymphocytes (10) and nonspecifically activated macrophages (4, 5). Antigen-specific, class I-restricted T lymphocytes of the $CD8^+$ subset were also found to play a role, possibly through their production of IFN-γ (11) or by virtue of their cytotoxic function (42).

The present study was undertaken to clarify the mechanisms (both cellular and cytokine mediated) leading to the onset of systemic protective immunity to *C. albicans* following persistent infection of mice with low-virulence variant cells. We report here on the results obtained with two chemically mutagenized, agerminative variants of a parent *C. albicans* strain. The two variants, although endowed with reduced pathogenicity in vivo, differed in the ability to induce protective anticandidal immunity and resulted in preferential activation of different T-cell subsets. This may suggest an important involvement of regulatory mechanisms in long-term colonization of mice with *C. albicans*.

MATERIALS AND METHODS

Mice. Hybrid (BALB/cCr × DBA/2Cr)F_1 (CD2F1; $H\text{-}2^d/H\text{-}2^d$) mice were obtained from Charles River Breeding Laboratories, Calco, Milan, Italy. Mice of both sexes, ranging in age from 2 to 4 months, were used.

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Yeast strains and infections. The origin and characteristics of the parent *C. albicans* (CA-6) strain have already been described in detail (10, 11). Clonal avirulent lines (Vir^-) of CA-6 were obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by incubating 10^8 cells per ml with the drug at 150 μg/ml for 90 min at 30°C (25), followed by centrifugation and suspension in distilled water. The cells were spread on yeast extract-peptone (YEP) complete medium (45); after growth, the resulting clones (which amounted to approximately 3% of the initial population) were tested for auxotrophy by transfer onto the appropriate medium (31). Two clones, Vir^- 3 (prototroph) and Vir^- 13 (biotin auxotroph), were selected that showed assimilation and fermentation patterns typical of *C. albicans* (33), although Vir^- 3 had acquired the ability to assimilate glycerol and melezitose, and Vir^- 13 had lost the ability to assimilate trehalose (API 20C AUX System). Like parental cells, Vir^- 3 and Vir^- 13 reacted with polyclonal anti-*C. albicans* sera and belonged to serotype A (Iatron System, Tokyo, Japan). Unlike parental cells that gave rise to yeast mycelial conversion, Vir^- 3 and Vir^- 13 grew as a yeast and produced short pseudomycelium under conditions that promote germ-tube formation in vitro or in vivo. When injected intravenously (i.v.) into recipient mice in doses of up to 10^7 yeast cells, the mutant strains resulted, as a rule, in no lethality, although sustained colonization of different mouse organs (in particular, the kidney and brain) could be detected according to a previously described pattern (49). After prolonged in vitro or in vivo passaging, Vir^- 3 and Vir^- 13 retained their respective phenotypes, including the inability to germinate and reduced pathogenicity. A full taxonomic characterization of the mutants, inclusive of DNA biotype determination, is in progress.

Parental and mutant yeast lines were grown to stationary phase at 30°C under slight agitation at 5×10^4 cells per ml of YEP medium. Under these conditions, cultures yielded 1×10^8 to 5×10^8 cells per ml, and the organisms grew as an essentially pure yeast-phase population. At the stationary phase, cells were harvested by low-speed centrifugation ($1,000 \times g$), washed twice in phosphate-buffered saline (PBS), and diluted to the desired density to be injected i.v. into mice in a volume of 0.5 ml.

Antibodies and phenotype analysis. The following monoclonal antibodies (MAbs) were used in the positive or negative selection of lymphoid populations with a predetermined cell surface antigen phenotype, according to previously described procedures (10, 11): anti-CD8 (53-6.72), anti-CD4 (GK1.5), anti-Thy-1.2 (30-H12), and anti-$I\text{-}A^d$ (MK-D6). In the positive selection of $CD4^+$ or $CD8^+$ cells, B-cell-depleted splenic lymphocytes were incubated with rat anti-mouse anti-CD4 or anti-CD8 MAbs, respectively, prior to their placement in petri dishes coated with a goat anti-mouse immunoglobulin G (IgG) antiserum cross-reacting with rat antibody determinants. After the nonadherent cells were removed, the attached cells were recovered, yielding a population of >98% cells positive for the selected surface antigen. The phenotype of purified lymphocytes was assessed by immunofluorescence staining (10, 11).

Production of culture supernatants containing cytokine activity. Supernatants from mixed lymphocyte-*Candida* cultures were obtained as described previously (10, 11). Briefly, $CD4^+$ lymphocytes, positively selected from pools of spleen cells and suspended in RPMI 1640 medium supplemented with 0.5% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol, were cultured (2×10^5/well) with heat-inactivated CA-6 cells at a lymphocyte-to-yeast cell ratio of 80:1 in 96-well flat-bottomed microtiter plates in 0.2-ml volumes in the presence of irradiated (2,000 rad) plastic-adherent macrophages (2×10^4/well). After incubation at 37°C in a CO_2 incubator for 24 h, culture supernatants were harvested by centrifugation, pooled, and assayed for cytokine activity.

Bioassays for cytokine activities. The procedures for assaying cytokine activities have been previously described in detail (10, 11). Briefly, cell supernatants were tested in functional bioassays for different cytokine activities, and titers (in units per milliliter) were calculated by comparing the dilutions of test samples that gave half-maximal responses with standard, recombinant preparations of murine cytokines (Genentech Inc., South San Francisco, Calif.). Interleukin-2 (IL-2) and IL-4 activities were measured by their ability to sustain the growth of HT-2 cells (47), using the tetrazolium-based colorimetric assay. IL-6 activity was determined by its capacity to support the growth of the murine IL-6-dependent cell line 7TD1 (48), obtained through the courtesy of J. Van Snick. In the IL-2 and IL-4 bioassay, samples were tested in the presence of anti-IL-2 (S4B6) or anti-IL-4 (11B11) MAbs, such that residual activity after the addition of anti-IL-2 or anti-IL-4 antibodies could be attributed to the presence of IL-4 or IL-2, respectively (47). Controls included recombinant IL-2 and IL-4 assayed in the presence of anti-IL-2, anti-IL-4, or both MAbs. In selected experiments, a mixture of S4B6 and anti-IL-2 receptor (7D4) (39) MAbs were also employed to neutralize IL-2 activity. MAbs were used as hybridoma culture supernatants at dilutions found to inhibit 95% HT-2 growth-promoting activity of the recombinant cytokines.

Assay for IFN-γ activity. A double-sandwich enzyme-linked immunosorbent assay (ELISA) was performed to quantitate IFN-γ in cell culture supernatants, essentially as described by Moll and Röllinghoff (35). Flat-bottomed polyvinyl chloride microtiter plates were coated with anti-IFN-γ MAb R4-6A2 (20 μg/ml in PBS overnight at 4°C). Unsaturated binding sites were blocked with 2% bovine serum albumin (BSA) for 1 h at 37°C. Plates were then washed with buffer (PBS, 0.05% Tween 20), the samples, diluted 1:2 in PBS, were added, and the plates were incubated for 2 h at 37°C. Plates were then washed and biotin-labeled anti-IFN-γ MAb AN-18.17.24 in PBS (5 μg/ml, a generous gift of S. Landolfo, University of Torino, Turin, Italy) was added (1 h at 37°C). The plates were washed again, and wells were incubated with an avidin-alkaline phosphatase conjugate (Vector Laboratories, Burlingame, Calif.) in PBS for 30 min. After extensive washing, the substrate *p*-nitrophenylphosphate (1 mg/ml) in diethanolamine buffer was added, and the A_{405} in the wells was read 3 h later. Units of IFN-γ were calculated from a standard titration of murine recombinant IFN-γ (Genentech), with a lower limit of cytokine detection of 1 U/ml.

ELISA. A micro-ELISA procedure was used to quantitate specific antibodies in the sera of yeast-infected mice. Flat-bottomed plates were coated overnight at 4°C with 10^7 heat-killed CA-6 cells per well in 100 μl of 0.1 M bicarbonate buffer (pH 9.6). The wells were then saturated with 1% BSA in PBS for 1 h at 37°C. One hundred microliters of a dilution of test antisera (from a pool of three mice per group) in PBS supplemented with 0.5% BSA and 0.01% Tween 20 were added and incubated for 1 h at 37°C. After extensive washes, 1:1,000 dilutions (100 μl) of alkaline phosphatase-conjugated rabbit anti-mouse IgG, IgG2a, or IgG1 (Zymed Laboratories, San Francisco, Calif.) were added. After 1 h at room temperature, the plates were washed, 200 μl of substrate solution per well was added, and the plates were incubated

at 37°C for 1 h. The optical density of triplicate samples was read with an ELISA reader, using a 405-nm filter.

LD analysis of cytokine-producing CD4$^+$ cells. The frequency of precursor CD4$^+$ cells with the potential for cytokine secretion in the spleens of yeast-infected mice was determined by established protocols (35, 47). Before limiting dilution (LD) analysis, bulk cultures of splenic lymphocytes were subjected to one cycle of in vitro restimulation (for 4 days, as described above) and rest (for 6 days) (39). Limiting numbers of positively selected CD4$^+$ cells (from 1×10^2 to 32×10^2) were then cultured with inactivated CA-6 cells (10^4) in the presence of 2×10^5 irradiated macrophages, using 36 replicates in microtiter plates in 0.2-ml volumes. On day 4, supernatants were harvested and split into four aliquots for the determination of IL-2, IL-4, and IFN-γ activity. For concurrent determination of IL-2 and IL-4 activity, the first three aliquots were tested for HT-2 growth-promoting ability: each well was scored as positive or negative for cytokine by comparison with an arbitrary threshold set 3 standard deviations above the mean optical density level generated by a set of background wells set up with antigen-presenting cells and antigen but no CD4$^+$ cells. One aliquot was incubated without added antibodies, to test for IL-2 and IL-4 activities; a second aliquot was tested in the presence of anti-IL-2 MAbs (S4B6 and 7D4), while a third aliquot was treated with anti-IL-2 and anti-IL-4 MAbs. IL-2 activity was defined as the reduction of the HT-2 response in the presence of anti-IL-2 MAbs, while IL-4 activity was defined as HT-2 growth-promoting activity detectable in the presence of anti-IL-2 MAbs, but not in the presence of anti-IL-2 and anti-IL-4 MAbs. Estimates of precursor frequency for each cytokine activity were made by standard protocols for limiting dilution analysis (35, 47).

LD analysis of *Candida*-specific CD8$^+$ cytotoxic lymphocytes. The procedure for LD analysis of *C. albicans*-specific CD8$^+$ lymphocytes has been described elsewhere in detail (42). Briefly, limiting numbers of positively selected CD8$^+$ responder lymphocytes (from 1×10^2 to 20×10^2/well) were stimulated with 5×10^4 heat-inactivated *Candida* cells in the presence of 10^6 irradiated Thy-1$^-$ Ia$^-$ splenocytes, using 48 replicates in microtiter plates in a final volume of 0.15 ml of IL-2-containing Dulbecco-modified Eagle medium. Cultures were fed with fresh medium (0.05 ml) on day 3. On day 7, each microculture was harvested and split into two replicates, and microcultures were assayed for cytotoxic activity to ^{51}Cr-labeled macrophages that had engulfed *C. albicans*. As a homogenous population of macrophages, in vitro-cultured bone marrow macrophages were used as target cells (13). After 10 to 14 days in culture, macrophages (>95% viable) were pulsed overnight with inactivated CA-6 cells (5×10^4/ml), washed, and labeled with ^{51}Cr to be used as target cells in the cytotoxicity assay. Estimates of cytotoxic cell frequency were done by standard procedures (22, 42).

DTH assay. A direct assay system for measuring the delayed-type hypersensitivity (DTH) response to cell surface antigens was employed (10, 11) in which heat-inactivated CA-6 cells (2×10^6/0.04 ml of pyrogen-free saline) were inoculated into the footpads of control or immunized mice. In the DTH transfer experiments, CA-6 cells were injected into the footpads of naive mice that had been adoptively transferred, 24 h earlier, by i.v. injection of immune lymphocytes. 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 footpad weight (right hind) over that of the saline-injected (left hind) counterpart. Data are the means ± standard errors of 6 to 8 mice per group.

TABLE 1. Anticandidal resistance in mice infected with avirulent yeast cells

Treatment strain and day[a]	Result of CA-6 challenge[b]		Candidacidal activity[c]	Increase in footpad weight[d] (mg)
	MST	*D/T* (*n* = 10)		
Untreated control	3	10	29.9 ± 3.0	3.8 ± 0.5
Vir$^-$ 3				
3	5	10	31.0 ± 4.2	5.0 ± 1.8
7	15[e]	7	50.3 ± 2.6[e]	9.4 ± 1.2[e]
14	>60[e]	0	58.7 ± 4.1[e]	24.2 ± 1.4[e]
Vir$^-$ 13				
3	3	10	28.0 ± 3.2	4.9 ± 0.6
7	3	10	27.8 ± 5.1	10.3 ± 0.8[e]
14	2.5	10	32.9 ± 3.3	8.7 ± 0.6[e]

[a] Vir$^-$ 3 or Vir$^-$ 13 (10^6 cells per mouse) were given as a single i.v. injection the indicated number of days before systemic challenge with CA-6, or intrafootpad 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; *D/T*, number of dead mice at 60 days over total number of animals tested.

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

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

[e] $P < 0.01$ to 0.0001 (treated versus control mice).

CFU inhibition assay. Pooled spleen cells (5×10^5 in 0.1 ml per well) from 3 to 5 mice were infected with CA-6 cells (5×10^4 in 0.1 ml of suspension per well). After a 6-h incubation at 37°C in 5% CO_2, Triton X-100 (final concentration of 0.1%) was added to the wells and the plates were vigorously shaken. 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 (means ± standard errors), according to the formula: percent colony formation inhibition = 100 − [(CFU of experimental group/CFU of control culture) × 100]. Control cultures consisted of *C. albicans* incubated without effector cells (9).

Statistical analysis. Differences in the numbers of viable yeast units in the CFU inhibition assay were analyzed by the Student *t* test. The same test was performed on footpad weight increase values in the DTH experiments. The data reported are from representative experiments, unless otherwise stated. Experiments with similar results were performed three to six times.

RESULTS

Effect of Vir$^-$ yeast injection on anti-*Candida* resistance, candidacidal activity of spleen cells, and delayed-type footpad reaction to *Candida* antigens. CD2F1 mice inoculated with 10^6 live Vir$^-$ 3 or Vir$^-$ 13 cells at different times were either challenged i.v. with virulent CA-6 cells or injected intrafootpad with inactivated *C. albicans* to be assayed 24 h later for footpad swelling, as described in Materials and Methods. Additional groups of mice treated with the mutant strains served as donors of spleen cells to be reacted in vitro with *C. albicans* in a CFU inhibition assay that is known to measure phagocytic killing of the yeast by splenic effector macrophages (4, 9). Table 1 shows that Vir$^-$ 3 treatment conferred

TABLE 2. Th1 cytokine production by variant-immune CD4+ lymphocytes

Cytokine activity[a]	Th1 cytokine production[b] after the following no. of days postinfection:				
	3	7	10	14	20
Nonimmune					
IL-2	8	10	12	5	10
IFN-γ	0	3	5	2	0
Vir⁻ 3 immunized					
IL-2	364	694	1,242	1,420	1,840
IFN-γ	88	160	130	111	81
Vir⁻ 13 immunized					
IL-2	496	42	30	55	43
IFN-γ	45	10	5	3	0

[a] Heat-inactivated CA-6 cells were cocultured with a mixture of CD4+ and gamma-irradiated splenic adherent cells for 24 h prior to collection of supernatants.

[b] Vir⁻ 3 or Vir⁻ 13 (10^6 cells per mouse) were given as a single i.v. injection a number of days before spleen cells were harvested as a source of CD4+ lymphocytes. Supernatants were tested for cytokine activity as described in the text. Each value (in units per milliliter) represents the mean of three to five separate experiments, each involving duplicate measurements. Standard errors, usually less than 10% of the mean, have been omitted. Zero values indicate cytokine levels below the sensitivity limits of bioassays and ELISA.

TABLE 3. Th2 cytokine production by variant-immune CD4+ lymphocytes

Cytokine activity[a]	Th2 cytokine production[b] after the following no. of days postinfection:				
	3	7	10	14	20
Nonimmune					
IL-4	0	3	0	5	2
IL-6	2	4	0	0	0
Vir⁻ 3 immunized					
IL-4	0	0	20	15	8
IL-6	0	0	2	5	10
Vir⁻ 13 immunized					
IL-4	0	0	170	842	982
IL-6	0	15	70	520	789

[a] Heat-inactivated CA-6 cells were cocultured with a mixture of CD4+ and gamma-irradiated splenic adherent cells for 24 h prior to collection of supernatants.

[b] Vir⁻ 3 or Vir⁻ 13 (10^6 cells per mouse) were given as a single i.v. injection a number of days before spleen cells were harvested as a source of CD4+ lymphocytes. Supernatants were tested for cytokine activity as described in the text. Each value (in units per milliliter) represents the mean of three to five separate experiments, each involving duplicate measurements. Standard errors, usually less than 10% of the mean, have been omitted. Zero values indicate cytokine levels below the sensitivity limits of bioassays and ELISA.

considerable protection against microbial challenge when the latter was performed 7 to 14 days after vaccination, at a time when highly candidacidal effectors were found in the spleens of Vir⁻ 3-infected mice. Concurrent with maximal anti-*Candida* resistance (day 14) was the detection of strong reactivity to *Candida* antigens in the footpads of sensitized mice. Neither protective immunity nor enhanced candidacidal activity in the spleen was detected in mice sensitized 7 to 14 days earlier with Vir⁻ 13 cells, although significant DTH reactivity was present in these mice starting from day 7 postinfection. In experiments testing additional time points (data not shown), we found that the pattern of reactivity of day 14 remained essentially unchanged through days 15 to 21 after the injection of either mutant strain.

IL-2 and IFN-γ secretion by yeast variant-immune CD4+ cells. Analogous to the results obtained with another variant strain of *C. albicans* (9), these data suggested the involvement of the Th1 subset of CD4+ cells in the acquired resistance to the fungus induced by vaccination with Vir⁻ 3 cells. These cells produce macrophage-activating factors, mediate DTH reactivity in vivo, and are characterized by a typical cytokine secretion pattern (36). This pattern includes IL-2 and IFN-γ, but not IL-4 or IL-6 which are characteristic Th2 cytokines. To explore the possible involvement of Th1 cells in anti-*Candida* resistance in our system model, we analyzed IL-2 and IFN-γ production in vitro by using purified CD4+ cells from mice infected with either mutant yeast strain. At different times after the injection of Vir⁻ 3 or Vir⁻ 13 cells, CD4+ cells from infected mice were assayed for cytokine production in response to CA-6 antigen. Table 2 shows that as early as 3 days postinfection, CD4+ cells from mice injected with either mutant could release IL-2 and IFN-γ in response to *C. albicans* in vitro. However, on days 7 to 20, Vir⁻ 3-treated mice produced increasing amounts of the two cytokines, whereas production of IL-2 rapidly declined in mice infected with Vir⁻ 13. In the latter, little or no IFN-γ was apparently released by CD4+ cells on days 10 through 20. Essentially the same results were obtained when the mitogen concanavalin A was used in place of yeast antigen for the in vitro stimulation of CD4+ cells from mice infected with Vir⁻ 3 or Vir⁻ 13 cells (data not shown).

IL-4 and IL-6 production. Several lines of evidence suggest that differentiation or growth or both of Th1 cells might be inhibited during a strong Th2 response, and vice versa (46). To explore the possible occurrence of a Th2-like response in *Candida*-infected mice, CD4+ cells from mice treated with Vir⁻ 3 or Vir⁻ 13 were assayed for IL-4 and IL-6 production at 3, 7, 10, 14, and 20 days after infection. The data in Table 3 show that large amounts of IL-4 and IL-6 were produced in response to specific antigen by CD4+ cells from Vir⁻ 13-infected mice starting from day 10 postinfection. In contrast, no significant production of the two cytokines was detected at any time with lymphocytes from Vir⁻ 3-infected mice. Similar results were obtained when mitogen-stimulated lymphocytes from either type of infected mice were assayed for IL-4 and IL-6 production (data not shown).

***Candida*-specific antibodies in immunized mice.** The Th1 subset of CD4+ T cells is also known to be the major source of help for IgG2a antibody production, whereas Th2 cells provide help for the synthesis of other isotypes, including IgG1 (36, 46). The possible participation of these cells in the anticandidal immunity induced by vaccination with Vir⁻ 3 cells was further investigated by searching for specific antibodies in the sera of mice infected with either variant 10 days earlier. Figure 1 shows optical density values in an experiment in which sera were assayed for the presence of specific IgG, IgG2a, and IgG1 antibodies. It appears that high levels of IgG2a were present in the sera of Vir⁻ 3-primed mice and that the latter antibodies accounted for most of the total IgG fraction (Fig. 1A). When the examination was extended to mice infected with Vir⁻ 13, we found their sera contained higher levels of specific IgG, with a large proportion represented by the IgG1 fraction (Fig. 1B).

Frequencies of cytokine-secreting CD4+ cells in mice infected with either variant. LD analysis of freshly isolated lymphocytes provides information at the clonal level on the effector activities of the entire range of T-cell populations

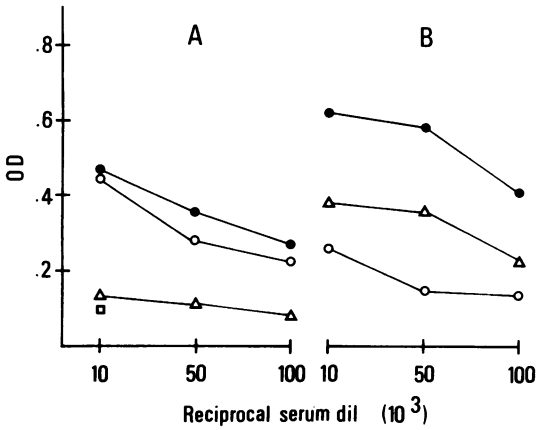

FIG. 1. Presence of *C. albicans*-specific IgG (●), IgG2a (○), and IgG1 (△) in sera of mice infected with Vir⁻ 3 (A) or Vir⁻ 13 (B) cells. Blood samples were obtained on day 10 and assayed by ELISA at different dilutions. IgG (□) levels in sera of intact mice. Standard errors, usually <1.5% of the mean, are not shown. OD, optical density; dil, dilution.

that become activated during infection and allows quantitation of antigen-specific precursor cells. Ten days after infection with Vir⁻ 3 or Vir⁻ 13 cells, splenic lymphocytes were collected and subjected to one cycle of in vitro restimulation and rest in bulk culture. This was followed by positive selection of CD4⁺ cells, LD analysis, and bioassay for IL-2 and IL-4 activity, as described in Materials and Methods. The production of IFN-γ was measured by ELISA. Figure 2 shows that the frequency of CD4⁺ cells producing IL-2 and

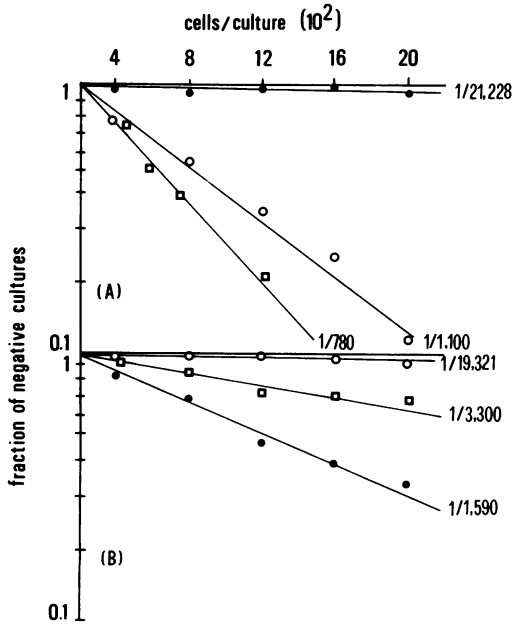

FIG. 2. Precursor frequencies of *C. albicans*-reactive CD4⁺ cells with the potential for production of IFN-γ (○), IL-2 (□), or IL-4 (●). Ten days after infection with Vir⁻ 3 (A) or Vir⁻ 13 (B) yeast cells, spleen cells were subjected to one cycle of in vitro restimulation and rest prior to selection of CD4⁺ cells and LD analysis. Numbers indicate the frequencies as estimated by the chi-square minimalization method.

IFN-γ was much higher in Vir⁻ 3-infected mice than in Vir⁻ 13-infected mice. In contrast, mice treated with Vir⁻ 13 showed a higher frequency of cells with the potential to secrete IL-4. CD4⁺ cells from uninfected mice gave negligible responses (data not shown).

Frequencies of antigen-specific CD8⁺ cells with cytotoxic activity to yeast-infected macrophages. We have recently shown that both CD4⁺ and CD8⁺ cells are required for optimal expression of acquired anticandidal resistance (11) and that the latter lymphocytes may acquire the potential to lyse yeast-infected macrophages after short-term exposure to IL-2 in vitro (42). Because stimulation of Th1 CD4⁺ cell activity is often associated with the activation of CD8⁺ lymphocytes (35), it seemed of interest to evaluate the frequencies of cytotoxic precursors in the spleens of mice infected with Vir⁻ 3 or Vir⁻ 13 *Candida* cells. Positively selected CD8⁺ lymphocytes were restimulated in vitro for 7 days with inactivated CA-6 cells in the presence of Thy-1⁻ Ia⁻ accessory cells and IL-2, prior to testing for cytotoxic activity to yeast-primed macrophages under LD conditions, as described previously (42). We found that the frequency of antigen-specific cytotoxic cell precursors was considerably higher in Vir⁻ 3-infected mice (1/5,028) than in Vir⁻ 13-infected mice (1/25,748). In contrast, precursor frequencies to unprimed target macrophages were similar, with values of 1/35,582 and 1/36,331 for mice infected with Vir⁻ 3 and Vir⁻ 13, respectively.

Transfer of DTH by CD4⁺ cells from mice infected with either variant. We have previously shown that DTH reactivity to CA-6 antigens can be conferred upon naive recipient mice by adoptive transfer of purified CD4⁺ cells from immunized donors (10). To further evaluate the possible contributions of CD4⁺ cells in anticandidal immunity following in vivo priming with Vir⁻ 3 or Vir⁻ 13 cells, we investigated the ability of such lymphocytes to transfer DTH reactivity to mice challenged intrafootpad with CA-6. Animals infected with either yeast variant (day 0) were treated on day 20 with a sublethal i.v. inoculum of CA-6 cells, and were used 1 week later as a source of CD4⁺ lymphocytes to be employed in the cell transfer experiments. Recipient mice received 10^7 purified CD4⁺ lymphocytes from either Vir⁻ 3- or Vir⁻ 13-infected mice; an additional group of mice received a mixture (2×10^7 cells per mouse) of the two lymphocyte populations. Controls included mice treated with nonimmune spleen cells or CD4⁺ cells from animals given only the boosting injection of CA-6 cells. Figure 3 shows the results. It is apparent that DTH reactivity was only transferable with CD4⁺ cells from Vir⁻ 3-infected mice. More interestingly, no significant footpad reaction was observed in mice receiving a mixture of cells from both types of yeast-infected donor mice.

DISCUSSION

In *C. albicans* infections, mechanisms against the yeast are believed to involve specific T cells, because patients with chronic mucocutaneous candidiasis, 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 systemic candidiasis, granulocyte function (43), cytokine production by natural killer and monocytic cells (14), the complement system (28) and humoral antibodies (32) may provide an essential first line of defense against the spread of the organisms, but specific cell-mediated immunity

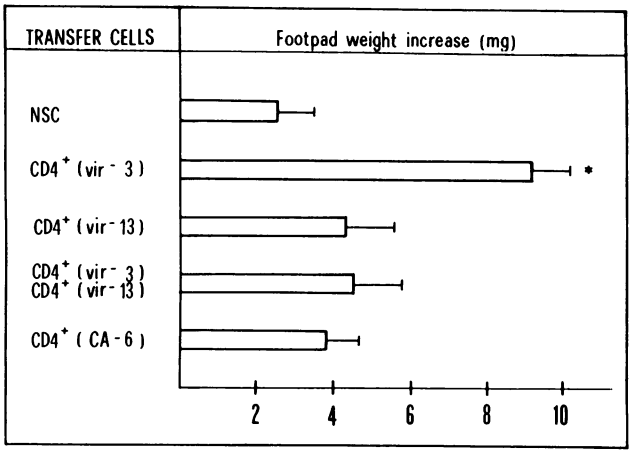

FIG. 3. Transfer of DTH by $CD4^+$ cells. Prospective recipients of an intrafootpad CA-6 challenge received $CD4^+$ cells from mice infected with Vir^- 3 or Vir^- 13, or a mixture of purified $CD4^+$ lymphocytes from these mice. The footpad weight increase was recorded 24 h after footpad challenge. Controls consisted of mice treated with nonimmune spleen cells (NSC) or $CD4^+$ cells from mice given only the boosting injection of CA-6 (see text for details). The asterisk indicates that $P < 0.001$ ($CD4^+$ cells versus NSC).

and other as yet unknown mechanisms may be important as well (6, 40). This condition may not be significantly different from that of laboratory animals in which innate resistance mechanisms (3, 9, 12, 23, 27, 30) tend to prevent the spread of the organisms until T-dependent immunity has developed sufficiently to help with clearance of the fungus (2, 8, 15, 20, 34). In agreement with this notion, we have recently shown that systemic colonization of mice with low-virulence *Candida* cells leads to prolonged stimulation of T cells (10) and activation of microbicidal macrophages that confer protection against an otherwise lethal *C. albicans* challenge (4). In this model system, we have demonstrated that both Th1 type $CD4^+$ and $CD8^+$ cells from immunized mice will release IFN-γ upon recognition of *Candida* antigen in vitro (11), are required for complete expression of anticandidal protection in vivo (11), and may exert synergistic, though possibly distinct, functions (42). The main objective of the present studies was to find out whether the sustained presence of low-virulence yeast cells invariably leads to induction of resistance in mice and whether immunoregulatory mechanisms may occur during infection. This issue is particularly interesting because immune abnormalities have long been known to occur in human (26) and murine (19) candidiasis.

By using mutagenized, attenuated variant cells of a parent *C. albicans* strain, we studied the development of specific immunity over a prolonged period of time, particularly focusing on correlation between onset of resistance and cytokine secretion by $CD4^+$ cells. Protective immunity was measured in vivo as susceptibility to challenge with parental cells, occurrence of DTH reactivity, and appearance in the serum of specific antibodies. In vitro, we analyzed the candidacidal function of splenic macrophages, production of Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-6) cytokines, precursor frequencies of cytokine-secreting cells, and development of antigen-specific cytotoxic activity by $CD8^+$ lymphocytes. The two mutants described in the present study (Vir^- 3 and Vir^- 13), although equally agerminative and avirulent, differed in their ability to trigger effective anticandidal responses and led to preferential activation of different $CD4^+$ cell subsets. In particular, the protective responses elicited by Vir^- 3 correlated with the production of large amounts of IL-2 and IFN-γ, whereas Vir^- 13 infection was associated with the release of Th2 cytokines. These results were confirmed by LD analysis of cytokine-secreting precursor cells in the spleens of 10-day-infected mice. Moreover, Vir^- 3 infection was associated with the occurrence of strong DTH reactivity, detection of specific IgG2a antibody in the sera, and presence in the spleen of activated macrophages and of $CD8^+$ precursor cells with the ability to lyse yeast-primed macrophages.

Several factors have been proposed to be potentially capable of influencing $CD4^+$ cell differentiation toward Th1 versus Th2 (24, 47). These include the antigen composition and dose, antigen-presenting cell type, lymphoid tissue microenvironment, route of immunization, and $CD4^+$ cell interaction during the clonal expansion or effector stage of response. The finding that Vir^- 3 infection resulted in induction of Th1 responses and development of anticandidal resistance can be taken to indicate that Vir^- 3 cells expressed high levels of antigens capable of triggering protective mechanisms. There is, of course, no evidence that mutagen treatment led to increased or de novo expression of immunogenic determinants on parental cells, as is true for mutagen treatment of many tumor cell lines in the mouse (7). In fact, many properties could contribute to the strong immunizing ability of Vir^- 3 cells, including reduced pathogenicity, persistence in the host and antigenic load in different mouse organs (29, 44, 49). However, comparative analysis of the properties of Vir^- 3 and Vir^- 13 cells allows for the conclusion that persistence of attenuated yeast-form *C. albicans* in the mouse does not result per se in the development of protective immunity. Moreover, the onset of resistance correlates with the detection of Th1-mediated responses, but Th2 cells may also be activated during infection. In Vir^- 13-infected mice, this was exemplified by the sustained production in vitro of IL-4 and IL-6, increased frequency of Th2 cytokine-producing cells, and occurrence of high titers of specific IgG1 antibody in the sera.

Several features of immune responses to microorganisms suggest that Th1 and Th2 cells are mutually inhibitory and that at least some of these effects are mediated by soluble cytokines (46). This inhibition may operate at the levels of effector functions, cytokine synthesis, and proliferation. IFN-γ, a Th1 product, inhibits the in vitro proliferation of Th2 but not Th1 clones (16, 18). IL-4, a Th2 product, supports the growth of Th2 cells in an autocrine manner in the presence of IL-1 (21). Th1 cells, which do not synthesize IL-4, show a more limited proliferative response to IL-4. In addition, IFN-γ is able to inhibit most of the effects of IL-4 on B cells, such as antibody class switching. A recently discovered Th2 cytokine, IL-10 (17), also has regulatory effects on Th1 cells. Responses composed largely of DTH, either with very low antibody production or antibodies mostly of the IgG2a isotype, may be regarded as Th1-mediated responses. Responses consisting of strong antibody responses and weak or absent DTH response are regarded as mediated by Th2 cells with minimal involvement of Th1 cells. Because cytotoxic T lymphocytes produce the Th1 set of cytokines, any response involving strong activation of these cells could give a selective advantage to the activation and growth of Th1 cells.

Although we are aware that caution is needed in discussing these preliminary results, it is tempting to speculate that both Th1 and Th2 cells may become activated in mice during infection with *C. albicans* and that these cells may exert

mutually inhibitory activities. This notion would be consistent with the apparent reciprocal regulation of Th1 and Th2 cytokine secretion patterns in mice infected with either variant and with the finding that $CD4^+$ cells from Vir^- 13-infected mice could apparently inhibit transfer of DTH response by Vir^- 3-immune lymphocytes. In this regard, it should also be noted that the extent of DTH response was similar in mice infected with Vir^- 3 or Vir^- 13 during the early stages of infection and that there was an inverse correlation between magnitude of footpad reaction and production in vitro of Th2 cytokines.

Anti-*Candida* suppressor T-cell activity in humans has been described by several investigators, often implying yeast-released mannan as an important cause of suppression (for a recent review, see reference 19). Garner et al. (19) showed that mannan administered i.v. to naive mice induced a population of suppressor T lymphocytes which, when transferred to immunized mice, suppressed antigen-specific DTH responses. Although $CD8^+$ cells appeared to be the main cells mediating suppression, removal of Lyt-1^+ lymphocytes also abrogated the ability of the transferred cells to induce suppression. Suppressor T cells stimulated by circulating antigen have also been described in other fungal systems, in particular murine cryptococcosis (37). In this model system, a cascade of a suppressor T cells was indeed found to be initiated by lymphocytes bearing the Lyt-1^+2^- surface antigen phenotype. How our present findings relate to such previous observations remains to be determined.

In conclusion, the results of the present study indicate that sustained colonization of mice with *C. albicans* may result in activation of Th1 $CD4^+$ cells and that development of acquired resistance is associated with the detection of strong Th1 responses. In addition, Th2 cells may also be activated during infection. The role of Th2 cell activation in candidal infections remains to be clarified.

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