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Effects of Cyclophosphamide on Murine Candidiasis

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Male CBA/J mice were given a single dose of 200 mg of cyclophosphamide (CY) per kg 3 days before a first or second cutaneous inoculation with viable *Candida albicans* in an attempt to suppress antibody formation and determine the effects of such suppression on the development of acquired immunity. After cutaneous inoculation, mice not treated with CY developed acquired immunity to intravenous challenge, which was accompanied by the development of circulating antibodies, delayed hypersensitivity, and in vitro responsiveness of lymph node cells to *Candida* antigens. CY treatment resulted in an immediate depression of peripheral blood leukocytes, with polymorphonuclear leukocytes and monocytes rebounding quickly to normal or above normal levels while lymphocytes remained depressed throughout the 4-week observation period. In vitro stimulation of lymph node cells from CY-treated mice was depressed shortly after treatment; however, responses to phytohemagglutinin and three *Candida* antigens (a cell wall preparation, a membrane preparation, and soluble cytoplasmic substances) recovered, whereas the responses to lipopolysaccharide did not. CY effects on the cutaneous lesion were twofold; first, the number of viable *Candida* cells in the lesions was much higher in animals receiving CY 3 days before *Candida* inoculation, and second, the size of the dermal lesion was either greatly enhanced or reduced depending upon the time of CY treatment relative to the number of cutaneous *Candida* inoculations. CY-treated animals developed higher levels of delayed hypersensitivity to the membrane preparation when infected once cutaneously than did corresponding untreated animals. The number of mice responding with circulating antibodies to soluble cytoplasmic substances after cutaneous inoculation was greatly reduced in CY-treated groups, and this impaired ability to produce antibodies correlated with the poor survival of these mice after intravenous challenge. Our results suggest that the ability to produce antibody at the time of challenge is crucial to successful defense against systemic candidiasis in this murine model.

Candida albicans is a member of the normal microbial flora of humans which lives commensally with its host until some precipitating event creates an environment favorable for invasion; examples of such events include trauma, pregnancy, diabetes mellitus, antibiotic therapy, immunosuppression, and underlying disease resulting in the impairment of the ability of a host to mount adequate immune responses (19, 20, 36). Based largely upon the relationship of candidiasis with the last of these examples, it has been widely conjectured that cell-mediated immunity is responsible for resistance in a normal host, and the possibility that the humoral system may be of equal or greater importance has received little consideration.

Using experimental animal models, Cutler (7), Rogers et al. (32), and Giger et al. (14) found that innate resistance, as opposed to acquired resistance, is not dependent upon the presence of functional T-lymphocytes and, by inference, upon cell-mediated immunity. However, Giger and his associates did find that the development of acquired resistance (i.e., resistance to reinfection) required the presence of functional T-lymphocytes in that thymectomized mice failed to develop resistance to reinfection. It was not determined, however, whether resistance to reinfection in normal mice was due to T-helper activity in association with antibody production or to T-effector function in a purely cellular immune phenomenon.

In an attempt to explore further the role of lymphocyte classes in acquired immunity to candidiasis, it was decided to modify animals in such a way as to reduce their B-cell populations, and thus antibody production, while at the same time enhancing certain aspects of their cellular immune systems. Cyclophosphamide (CY) was chosen as the agent of immunosuppression because, with proper doses and appropriate timing, this immunosuppressant potentiates delayed hy-

persensitivity to sheep erythrocytes (15, 21) or microbial antigens (12, 17) while selectively depleting B-lymphocytes and T-helper cells (23, 35). Since a model of cutaneous candidiasis has been used in this laboratory previously (10, 13, 14) and is reasonably well defined, our efforts were directed toward an investigation of the effects of CY upon the immune response(s) to *C. albicans* using that model, with the intent of gaining some insight into a possible role of B-lymphocytes in acquired resistance to candidiasis.

MATERIALS AND METHODS

Cultural and cell fractionation techniques. *C. albicans* B311 (serotype A; originally obtained from H. Hasenclever) was maintained by monthly transfer on glucose-peptone agar slants and stored at 4°C. Most of the cultural and fractionation techniques used in these studies have been reported elsewhere (9, 10, 14).

Viable blastospores either for inoculation into mice or for preparation of subcellular fractions were grown in soy dialysate broth (31) for 18 h at 37°C on a gyratory shaker. To obtain subcellular fractions, washed blastospores were disrupted in a Braun model MSK homogenizer (Bronwill Scientific Inc., Rochester, N.Y.), and fractions rich in cell walls, membranes and mitochondria, or soluble cytoplasmic substances were obtained after a series of three differential centrifugations, beginning with 400 × *g* and ending with 144,000 × *g*. The cell wall fraction was extracted with ethylenediamine to obtain water-soluble glycoproteins (9). The soluble cytoplasmic substance fraction was dialyzed (retention, ≥10,000 daltons) to remove low-molecular-weight components, lyophilized, and not fractionated further. The membrane-mitochondrion fraction was treated essentially as described previously (10), with one modification. Before extraction with hot phosphate-buffered saline (pH 7.4), the dry membrane-mitochondrion fraction was treated with 1-butanol according to the method of Cohen and Warringa (4, 29). Butanol was added to the membrane-mitochondrion fraction (4 mg of membrane-mitochondrion fraction per ml of 1-butanol), and the mixture was held in an ice bath for 20 min with continuous stirring. The mixture was allowed to stand undisturbed for an additional 10 min and then centrifuged at 400 × *g*. The supernatant was discarded, and the pellet was dried under a stream of nitrogen, stored in a desiccator overnight to remove traces of butanol, and then extracted with hot phosphate-buffered saline (10). After recovery of protein-rich material from the phosphate-buffered saline extract by precipitation from saturated ammonium sulfate solution, the modified hot extract (HEX) (referred to throughout this paper as B-HEX) was stored at −20°C. The protein and carbohydrate contents of HEX, the water-soluble glycoprotein, and the soluble cytoplasmic substances have been reported previously (10), and B-HEX does not differ from HEX in that B-HEX contains approximately 75% protein and 25% carbohydrate.

Preparation of CY-treated mice. Male CBA/J mice 10 to 12 weeks old (Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally with saline alone or with 200 mg of CY (Cytoxan; Mead Johnson Laboratories, Evansville, Ind.) dissolved in saline per kg.

Experimental design. The time of CY injection varied with the protocol (Table 1), but in all cases preceded a cutaneous inoculation of viable *C. albicans* by 3 days. In some animals (CY1°) this represented the only exposure of the animals to *Candida*, but one group (CY1°2°) received a second cutaneous inoculation 2 weeks later and a third group (1°CY2°) was inoculated with *Candida* 11 days before as well as 3 days after CY administration. In both CY-treated and untreated mice, groups of animals inoculated at the same time with CY and/or *Candida* were evaluated separately for delayed hypersensitivity, lymphocyte stimulations, antibody response, and resistance to intravenous challenge in order to eliminate any possible influence of one determination on another.

Evaluation of the effects of CY treatment. Total peripheral leukocytes were determined on groups of five normal or CY-treated mice by using a Coulter counter (model ZF), differential leukocyte counts were performed on Wright stained smears of the blood, and hemoglobin levels were determined by using a Coulter hemoglobinometer. Body weights for each mouse were recorded over the duration of the experiment.

Inguinal lymph node cells from mice treated with CY as well as from untreated mice were assessed for responses to purified phytohemagglutinin (PHA; Wellcome Reagents Ltd., Beckenham, England) and lipopolysaccharide B (*Salmonella typhosa* O901; Difco Laboratories, Detroit, Mich.) at selected times during the experiment (Table 1). Lymph nodes were excised and teased apart in cold RPMI 1640 medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and supplemented with 20 mM L-glutamine, streptomycin (100 μg/ml), penicillin (100 μg/ml), 5% (vol/vol) heat-inactivated horse serum, and 5×10^{-6} M 2-mercaptoethanol (1). All components were obtained from GIBCO Laboratories, Grand Island, N.Y., with the exception of 2-mercaptoethanol, which was obtained from Calbiochem, La Jolla, Calif. Lymph node cells from three mice were pooled, and the suspensions were adjusted to 2.5×10^6 viable nucleated cells per ml as determined by trypan blue exclusion. Stimulations were performed in triplicate in Microtest II plates (Falcon Plastics, Oxnard, Calif.), with each well receiving 0.1 ml of the cell suspension and 0.1 ml of either complete medium or medium containing mitogen. Cultures were incubated at 37°C in a humid atmosphere of 5% CO_2 and pulsed after 48 h with 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mg; New England Nuclear Corp., Boston Mass.) contained in 10 μl of sterile nonpyrogenic saline, and the cells were harvested 18 h later by using a MASH II unit (Microbiological Associates, Bethesda, Md.). After drying and suspension in a liquid scintillation cocktail, the samples were counted in a Beckman LS-250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The results were expressed as mean counts per minute and mean counts per minute of experimental cultures divided by mean counts per minute of controls.

Evaluation of responses to infection with *C.*

TABLE 1. *Experimental design for treatment with CY, inoculation with C. albicans, and in vivo and in vitro assays of immunity*

Group	Treatment on day:[a]							
	−3	0	11	14	17	21	27	28
No CY								
One cutaneous inoculation (1°)				Cut.	LS	FPT, LS, or IV	CIE	FPT, LS, or IV
Two cutaneous inoculations (1°2°)		Cut.		Cut.	LS	FPT, LS, or IV	CIE	FPT, LS, or IV
Cy-treated								
CY before one cutaneous inoculation (CY1°)			CY	Cut.	LS	FPT, LS, or IV	CIE	FPT, LS, or IV
CY before two cutaneous inoculations (CY1°2°)	CY	Cut.		Cut.	LS	FPT, LS, or IV	CIE	FPT, LS, or IV
CY between two cutaneous inoculations (1°CY2°)		Cut.	CY	Cut.	LS	LPT, LS, or IV	CIE	FPT, LS, or IV

[a] Abbreviations: Cut., Intracutaneous inoculation with 10^6 viable *C. albicans* blastosphores; LS, lymphocyte stimulation assay; FPT, footpad test; CIE, animals were bled and counterimmunoelectrophoresis with *C. albicans* soluble cytoplasmic substances and sera was performed; IV, intravenous inoculation with 10^4 viable *C. albicans* blastosphores.

albicans. Mice were inoculated and evaluated as described previously (10, 13). Briefly, 10^6 viable *C. albicans* blastospores suspended in 0.5 ml of nonpyrogenic saline were inoculated intradermally on the shaved lower abdomen. When animals were inoculated twice, the second inoculation was on the opposite side. Discrete lesions developed and were measured, and their volumes were calculated as previously described (13), using groups of 15 animals. Other mice, in groups of three, were used for the quantitative culture of dermal lesions. Such lesions were cultured 3, 7, or 14 days after one or two cutaneous inoculations with *Candida*. The lesions were excised in toto and ground in a mechanized tissue grinder, and varing dilutions were plated in triplicate onto Sabouraud agar containing chloramphenicol. Kidneys were also removed from these animals, homogenized, and plated.

At 7 or 14 days after the first or second cutaneous inoculation with *C. albicans*, groups of 10 mice were footpad tested (5, 6) with 20 μg of protein of B-HEX in 20 μl of nonpyrogenic saline. The net increase in footpad thickness (13) was determined 0.25, 4, 7, 24, and 48 h after antigen injection.

Inguinal lymph nodes draining the site of cutaneous inoculation with *C. albicans* were excised from three mice per group and pooled, and a single cell suspension was prepared in cold RPMI 1640 medium as described above for mitogen stimulations. *Candida* antigens (B-HEX, water-soluble glycoproteins, and soluble cytoplasmic substances) were added to cells (2.5×10^5 cells per well) at optimal stimulatory concentrations, as determined in preliminary experiments, and cultures were pulsed with [^{3}H]thymidine on the day 3 of incubation and harvested 18 h later.

Sera obtained by bleeding mice from the retroorbital venous plexus 14 days after the first or second inoculation with *Candida* were assayed for antibodies to soluble cytoplasmic substances by using counterimmunoelectrophoresis (16, 30). Resistance to reinfection was assessed at the same time by challenging groups of 15 mice intravenously with 10^4 viable blastospores. Deaths were recorded over an 8-week period, and the survival data were analyzed by a life table method (3, 8). At the end of 8 weeks, survivors were sacrificed, and their kidneys were removed, homogenized, and plated.

Statistical analysis. Results other than survival data were analyzed by using Student's *t* test.

RESULTS

Evaluation of CY-treated animals. The weight range for untreated animals was 24 to 26 g, whereas that for the CY-treated animals was 21 to 24 g. Weight loss occurred within 1 week of administration of CY and remained relatively constant throughout the experiment. The hemoglobin range for untreated mice was 13 to 15 g/dl, whereas that of CY-treated animals was 11 to 13 g/dl.

Total peripheral blood leukocytes were severely depressed after administration of CY, but the effects of CY on the different cell types was not equal. Representative responses are shown in Fig. 1. The number of polymorphonuclear leukocytes (PMN) was significantly lower ($P < 0.05$) in CY-treated mice on day −1 (2 days after the injection of CY) compared with untreated animals. The number of PMNs in CY-treated mice increased to normal levels by day 2 and

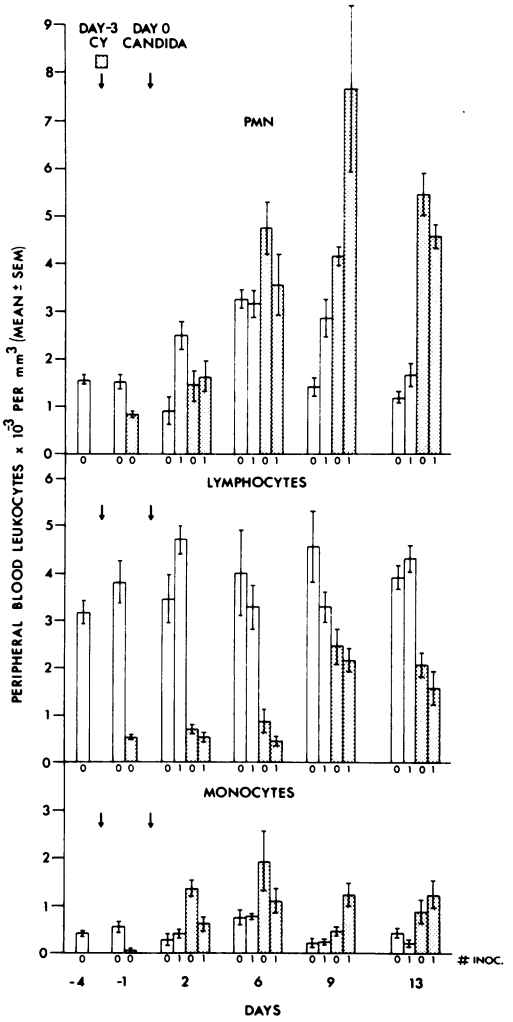

FIG. 1. *Differential counts on peripheral blood leukocytes. Open bars represent counts in mice not receiving CY, and stippled bars represent mice treated with CY on day −3. Numbers directly beneath each bar designate animals given one (1) inoculation with 10^6 viable C. albicans or uninoculated mice (0). Each value was determined from data on 5 to 15 mice. SEM, standard error of the mean.*

above normal levels by day 9 ($P < 0.02$) and day 13 ($p < 0.001$), returning to normal values by day 27 (data not shown). Although on several days (days 2, 9, 16, and 27) infected mice which had not been treated with CY had significantly more PMNs than the corresponding uninfected mice, only on one occasion was there such a difference between PMN levels of infected and uninfected mice treated with CY (day 16) (data not shown). The number of circulating lymphocytes in CY-treated mice was also severely depressed on day 2 but, unlike PMNs, remained depressed throughout the observation period, increasing to near normal but still below normal levels only on day 9. On day 23 (data not shown) uninfected animals (both CY treated and untreated) had more lymphocytes than did twice-infected animals. Monocyte levels in CY-treated mice were also severely depressed on day −1 ($P < 0.001$); however, they rebounded to normal or above normal values at all subsequent measurements. A significant difference between monocyte levels of infected and uninfected CY-treated mice was detected on days 2 and 9. Although not shown in Fig. 1, on day 27, just before intravenous challenge with *C. albicans*, CY-treated mice had normal levels of PMNs and monocytes and below normal levels of circulating lymphocytes.

When mitogens were used to stimulate lymphocyte proliferation, the lymphocytes from CY-treated mice showed the most pronounced and sustained reductions in response to lipopolysaccharide. There was, however, also some effect on responsiveness to PHA as well (Table 2) 6 days after injection of CY (day 17). Although PHA responses of the various groups were somewhat erratic, it was evident that, unlike lipopolysaccharide responses, the response to PHA had recovered in CY-treated mice by day 28.

Characteristics of cutaneous lesions after CY treatment. The volumes of the lesions in CY-treated and untreated mice receiving either one or two cutaneous inoculations with viable *C. albicans* blastospores are shown in Fig. 2. Animals not receiving CY but inoculated a second time cutaneously with *Candida* (1°2°) developed lesions that were significantly larger than those which developed in response to a first inoculation with *Candida* (1°), whereas animals receiving CY before a first infection (CY1°) or between the two cutaneous inoculations (1°CY2°) developed lesions that were much larger than those of non-CY-treated animals. The increased volume may have been due, at least in part, to increased multiplication of the organism within the lesion, since lesions cultured immediately after CY treatment, irrespective of whether that treatment preceded a first or second inoculation with *Candida*, contained significantly larger numbers of organisms than observed in animals not treated with CY (Fig. 3). Those animals that were given CY before a first inoculation and then inoculated a second time 2 weeks later (CY1°2°) developed lesions in response to the second inoculation that were much smaller than those in other CY-treated groups or in twice-infected non-CY-treated mice (1°2°). The dermal lesions in CY1°2° mice were, in fact,

TABLE 2. *Tritiated thymidine uptake by lymph node cells from untreated or CY-treated mice in response to lipopolysaccharide or PHA assessed in uninoculated mice or after one or two cutaneous inoculations with viable C. albicans blastosphores*

Day[a]	CY treatment	No. of cutaneous inoculations	[^{3}H]thymidine uptake after the following treatments:		
			Unstimulated (mean cpm)	Lipopolysaccharide (50 μg/well) (E/C)[b]	PHA (0.05 μg/well) (E/C)
17					
	No	None	479 (12)[c]	11.6 (1.0)	184.7 (5.0)
	No	1	1,549 (207)	11.8 (1.9)	57.8 (7.0)
	No	2	1,167 (110)	11.9 (2.0)	67.4 (5.7)
	Yes	None[d]	705 (130)	9.2 (1.5)	102.1 (13.6)[e]
	Yes	1[f]	9,846 (1,223)	0.7 (0.4)[e]	7.1 (1.0)[e]
	Yes	2[g]	6,036 (711)	0.5 (0.2)[e]	9.4 (1.8)[e]
	Yes	2[h]	3,293 (294)	1.6 (0.1)[e]	46.4 (4.9)[e]
28					
	No	None	2,333 (42)	14.9 (0.4)	24.6 (1.3)
	No	1	829 (169)	10.9 (1.9)	122.0 (26.1)
	No	2	1,189 (61)	17.0 (0.6)	56.0 (4.6)
	Yes	None[d]	1,926 (280)	3.3 (0.5)[e]	41.3 (6.6)
	Yes	1[f]	3,597 (470)	1.1 (0.2)[e]	33.9 (5.2)[e]
	Yes	2[g]	2,854 (1,278)	2.1 (0.6)[e]	45.6 (15.8)
	Yes	2[h]	ND[i]	ND	ND

[a] See Table 1.
[b] C/E, Counts per minute in experimental cultures divided by counts per minute in unstimulated controls.
[c] Numbers in parentheses are standard errors.
[d] CY was given on day 11.
[e] Value significantly decreased ($P < 0.05$) compared with the corresponding untreated control animals.
[f] CY was given 3 days before a first inoculation (CY1°).
[g] CY was given 3 days before a second inoculation (1°CY2°).
[h] CY was given 3 days before the first of two inoculations (CY1°2°).
[i] ND, Not done.

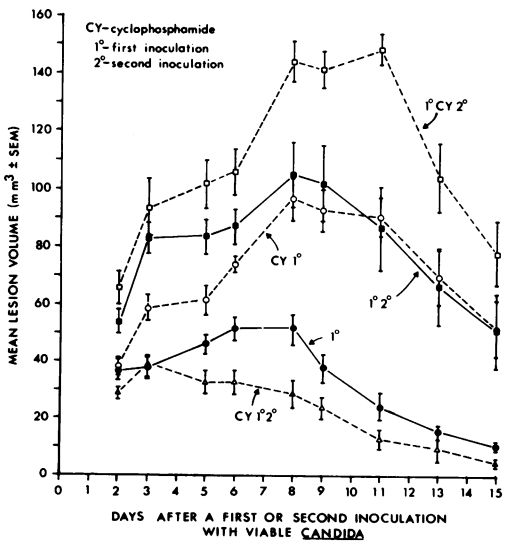

FIG. 2. *Lesion volumes in CY-treated or untreated mice after a first or second cutaneous inoculation with 10^6 viable C. albicans blastospores. SEM, standard error of the mean.*

similar to but smaller than those induced in non-CY-treated mice given a single inoculation (1°). It appears then that lesion size does not reflect the number of viable *Candida* cells present or the ability of the host to clear the cutaneous infection (Fig. 2 and 3) but is related to an as yet-undefined host response to the presence of *Candida* in the skin.

When the kidneys from mice not treated with CY were cultured after one or two cutaneous inoculations with *C. albicans*, they were uniformly negative. On the other hand, mice treated with CY 3 days before their first or second inoculation with *Candida* had small numbers of *Candida* in their kidneys at the 3- and 7-day observation periods, but were culture negative by day 14. The effects of CY on dissemination were not long lasting, however, in that the kidneys of mice treated with CY before the first two cutaneous inoculations with *Candida* were culture negative at all observation periods after the second inoculation. This indicated that although systemic spread occurred as a result of CY treatment, the animals were apparently able to control multiplication readily.

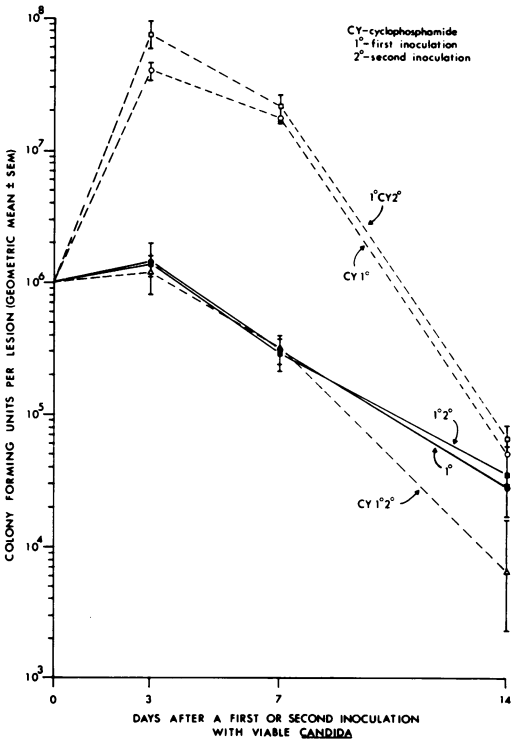

FIG. 3. *Colony-forming units of Candida per lesion in CY-treated or untreated mice inoculated once or twice cutaneously with 10^6 viable C. albicans blastospores. SEM, standard error of the mean.*

In vivo responses to *Candida* antigens. Animals treated with CY were footpad tested 7 days after one or two cutaneous inoculations with *Candida*, and their responses were compared with those in mice not treated with CY. Animals treated with CY developed high levels of delayed hypersensitivity to the membrane antigen B-HEX, such levels being comparable to those seen in animals infected cutaneously twice and never treated with CY (1°2°) (Fig. 4). It should be noted that one inoculation with *Candida* 3 days after CY treatment (CY1°) resulted in a twofold enhancement of delayed hypersensitivity as compared with mice not receiving CY but inoculated once with *Candida* (1°). Furthermore, when CY was administered between two cutaneous inoculations with *Candida* (1°CY2°), the CY did not interfere with the booster effect on delayed hypersensitivity normally observed in mice given a second inoculation with *Candida* (1°2°), indicating that the effector cells for delayed hypersensitivity to *Candida* are not sensitive to CY treatment when administered either 3 days before exposure to *Candida* or 11 days after a primary inoculation. In these experiments footpad testing was performed 7 days after a first or second cutaneous inoculation at a time when peak delayed hypersensitivity was present in mice not treated with CY (10).

Since mice were challenged intravenously 14 days, as well as 7 days, after the first or second cutaneous inoculation with *Candida*, mice treated with CY at various times with respect to inoculation with *Candida* were tested for delayed hypersensitivity at 14 days in the same experiment. The data presented for day 7 in Table 3 are comparable, therefore, to the data presented for CY-treated mice in Fig. 4. Furthermore, as has been observed previously with mice not treated with CY (10), the level of delayed hypersensitivity in mice treated with CY 3 days before either a first or second cutaneous inoculation with *Candida* (CY1°, 1°CY2°) was substantially reduced compared with that observed at 7 days. On the other hand, there was little difference between the 7- and 14-day reactions in mice which had been treated with CY before the first inoculation with *Candida* and were then footpad tested after a second inoculation with *Candida* (CY1°2°).

In contrast to delayed reactions, immediate hypersensitivity responses (15 min) in CY-treated mice were either unaffected (1°CY2°, CY1°) or slightly reduced (CY1°2°) when compared with untreated animals. The 15-min response is presumably antibody mediated (immunoglobulin E or immunoglobulin G1 or both), yet unlike circulating antibody (see below), it was unaffected by CY treatment.

Since animals treated with CY before a first cutaneous inoculation with *Candida* had approximately 100-fold more viable *Candida* cells in their lesions (Fig. 3) than non-CY treated controls, we reasoned that increased antigenic stimulation and not CY may have contributed to the enhanced delayed-type hypersensitivity responses observed in CY-treated animals. Therefore, untreated mice were inoculated cutaneously once with either 10^6 or 10^8 viable blastospores of *C. albicans* and footpad tested with B-HEX 7 days later. Animals inoculated with the larger dose (10^8) responded with levels of delayed hypersensitivity that were consistent with the levels observed in CY-treated animals, with a mean net increase in footpad thickness of 0.92 mm (standard error of the mean, 0.06) whereas those infected with the lower dose (10^6) had a mean net increase in footpad thickness of 0.46 mm (standard error of the mean, 0.08). It could be, therefore that increased proliferation

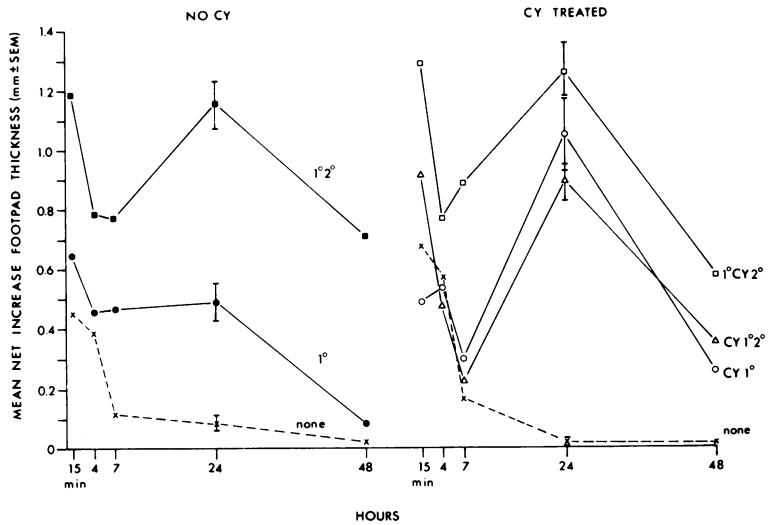

FIG. 4. *Footpad responses to 20 μg of B-HEX protein in CY-treated or untreated mice inoculated once or twice cutaneously 7 days previously with 10^6 viable C. albicans blastospores. SEM, standard error of the mean.*

TABLE 3. *Mean net increase in footpad thickness 24 h after the injection of C. albicans B-HEX into CY-treated mice*

Group	Mean increase in footpad thickness (mm) on day:[a]	
	7	14
CY	0.11(0.02)[b]	0.13(0.03)
CY1°	1.51(0.10)	0.82(0.08)
1°CY2°	1.38(0.09)	0.85(0.11)
CY1°2°	0.87(0.10)	0.69(0.08)

[a] Number of days after one or two cutaneous inoculations with *C. albicans*.

[b] Numbers in parentheses are standard errors of the mean.

of *Candida* was at least partially, if not wholly, responsible for the observed enhancement in footpad swelling.

In vitro responses to *Candida* antigens. Lymph node cells from infected mice were responsive to all three *Candida* antigen preparations at one time or another, and it should be noted that the responses of cells from CY-treated mice were affected in a manner similar to the PHA responses presented in Table 2. That is, on day 17 (data not shown) the response to *Candida* antigen was severely depressed, but by day 21 (Table 4) antigen responsiveness had begun to recover and had fully recovered by day 28 (data not shown). Lymphocytes from mice treated with CY before the first of two inoculations with *Candida* were capable of normal responses on all days tested. Most notable of the day 21 responses was the absence of an enhanced in vitro stimulation by animals given CY 3 days before their first cutaneous inoculation with *Candida* (CY1°). This was in contrast to the observation that animals treated in a similar manner but footpad tested on day 21 had enhanced levels of delayed hypersensitivity. On day 28, at the time of intravenous challenge with *Candida*, in vitro responses to *Candida* antigens by lymph node cells had fully recovered.

Animals treated with CY and infected with *Candida* responded poorly with antibody formation demonstrable with soluble cytoplasmic substances (Table 5). No more than 8% of the CY-treated animals had detectable circulating antibody 14 days after one or two cutaneous inoculations with *Candida*, whereas 83% of those animals inoculated twice but never treated with CY had antibody.

Intravenous challenge of CY-treated and untreated mice. Animals not treated with CY but inoculated once or twice cutaneously with *C. albicans* before intravenous challenge developed resistance to reinfection, as evidenced by the fact that 61 and 67% were still alive 8 weeks after intravenous challenge, whereas only 27% of those animals not inoculated cutaneously before intravenous challenge survived (Table 5). Moreover, all of the survivors in the group which had neither received CY nor been previously infected cutaneously with *Candida* had *Candida* demonstrable within their kidneys. Approximately 50% of those animals previously infected cutaneously were able to rid themselves of the organism. CY-treated animals, on the other hand, regardless of the timing of the CY treatment, survived poorly

TABLE 4. *Tritiated thymidine uptake by lymph node cells from mice 7 days (day 21) after one or two cutaneous inoculations with viable C. albicans*[a]

CY treatment	No. of cutaneous inoculations	[^{3}H]thymidine uptake after the following treatments:			
		Unstimulated (mean cpm)	B-HEX (20 μg/well) (E/C)[b]	Water-soluble glycoprotein (200 μg/well) (E/C)	Soluble cytoplasmic substances (100 μg/well) (E/C)
No	None	547 (172)[c]	1.5 (0.5)	1.0 (0.3)	2.0 (0.5)
	1	800 (102)	21.6 (7.2)	13.3 (3.7)	30.6 (3.4)
	2	501 (59)	25.0 (5.8)	16.2 (0.2)	44.6 (2.2)
Yes	1[d]	3,432 (1,277)	1.7 (0.5)[e]	3.6 (1.0)	9.6 (2.6)[e]
	2[f]	7,306 (2,675)	5.2 (0.3)[e]	6.7 (1.8)[e]	11.4 (3.2)[e]
	2[g]	2,982 (1,273)	22.3 (5.3)	19.4 (4.2)	37.4 (9.8)

[a] Cells from untreated animals or animals treated with CY were stimulated with *C. albicans* B-HEX, water-soluble glycoprotein, and soluble cytoplasmic substances. Data were compiled from three separate experiments.
[b] E/C, Counts per minute in experimental cultures divided by counts per minute in unstimulated controls.
[c] Numbers in parentheses are standard errors.
[d] CY was given 3 days before a first inoculation (CY1°).
[e] Values significantly decreased ($P < 0.05$) compared with the corresponding untreated control animals.
[f] CY was given 3 days before a second inoculation (1°CY2°).
[g] CY was given 3 days before the first of two cutaneous inoculations (CY1°2°).

TABLE 5. *Effect of CY treatment on the formation of precipitins to a cytoplasmic preparation of C. albicans (soluble cytoplasmic substances) and on protection as judged by survival and kidney culture after intravenous challenge with 10^4 viable C. albicans cells*

CY treatment	No. of cutaneous inoculations	Before intravenous challenge		Eight weeks after intravenous challenge			
		Counterimmunoelectrophoresis		Kidney culture[a]		Survival	
		No. positive/total no.	% Positive	No. negative/total no.	% Negative	No. alive/total no.	% Survival
No	None	0/30	0	0/30	0	8/30	27
	1	8/44	18	12/28	57	17/28	61
	2	35/42	83	12/30	40	20/30	67
Yes	None[b]	0/23	0	2/30	7	3/30	10
	1[c]	1/42	2	2/29	7	5/29	17
	2[d]	2/27	5	3/28	11	3/28	11
	2[e]	3/36	8	1/22	5	2/21	10

[a] Animals with fewer than 25 colonies of *C. albicans* per kidney were considered negative.
[b] CY was given 3 days before the first inoculation, 11 days after the first inoculation, and 3 days before the second inoculation.
[c] CY was given 3 days before the first inoculation (CY1°).
[d] CY was given 11 days after the first inoculation and 3 days before the second (1°CY2°).
[e] CY was given 3 days before a first inoculation (CY1°2°).

in all instances, and most of the survivors had *Candida* recoverable from their kidneys. Furthermore, few CY-treated animals had demonstrable antibody to cytoplasmic antigens at the time of intravenous challenge, whereas significantly more non-CY-treated animals, especially those which were inoculated twice cutaneously, had antibody at that time.

As stated above, the intravenous challenges described above were performed 14 days after one or two cutaneous inoculations, a time when there was still considerable demonstrable delayed hypersensitivity in animals not treated with CY but infected twice cutaneously with *Candida* (10) and in animals treated with CY and also infected with *Candida* cutaneously (Table 3). The maximal delayed hypersensitivity response, however, occurred 7 days after infection, and since animals treated with CY and infected cutaneously only once with *Candida* (CY1°) developed levels of delayed hypersensitivity at that time which were comparable to the

levels in animals not treated with CY but infected twice cutaneously (1°2°), it was decided to compare their survival rates after intravenous challenge. Despite the fact that the CY1° animals had high levels of delayed hypersensitivity, they survived poorly over an 8-week period (27%) compared with the 1°2° animals (80%). The CY-treated and untreated control animals had survival rates similar to those of the CY1° animals (31 and 20%, respectively).

DISCUSSION

The net effect of CY treatment was to nullify the ability of mice to develop resistance to reinfection, as assessed by intravenous challenge after recovery from one or two cutaneous infections with *C. albicans*. Moreover, the lack of resistance to reinfection in CY-treated animals was observed in the presence of strong delayed hypersensitivity to the B-HEX antigen. CY altered the ability of mice to confine organisms to early cutaneous lesions and control multiplication as well, since some systemic spread occurred, as evidenced by the finding of small numbers of *Candida* cells in the kidneys of CY-treated mice several days after cutaneous challenge and by the finding of many more colony-forming units in the lesions of mice treated with CY 3 days before cutaneous inoculation. Such mice were, however, able to control systemic spread, and *Candida* could not be cultured from kidneys 14 days after cutaneous inoculation.

The lesions resulting from first and second cutaneous inoculations of untreated mice contained the same numbers of viable *Candida*, yet they varied greatly in size. Giger and associates (13) reported that lesions taken from such mice could not be distinguished on the basis of histopathology and later reported (14) that thymectomized mice did not respond with larger lesions as a result of a second cutaneous inoculation. In the present study, CY had two effects upon the cutaneous lesions. First, when administered 3 days before inoculation with viable *Candida*, many more viable *Candida* cells were cultured initially from the developing lesions than in untreated mice. By 14 days after inoculation, these CY mice had populations of *Candida* equivalent to all other groups (Fig. 3). Second, CY treatment resulted in either an enhancement or a depression of lesion size, which was unrelated to the numbers or organisms cultured from the lesions. The mechanisms responsible for the cutaneous reaction to an intradermal injection of live *Candida* remain unclear, yet appear to be immunologically based. In light of the frequency of cutaneous involvement in humans, this aspect of the model is in need of further investigation.

Other investigators working with CY in experimental models involving *C. albicans* have been concerned primarily with innate resistance to systemic challenge (18, 27) at a short interval after CY treatment, and on the basis of their experiments they concluded that phagocytic cells were necessary for innate defense against candidiasis. In our studies we were more concerned with acquired resistance, and the systemic challenge was designed to detect acquired resistance at a time when phagocytic cells, (both monocytes and PMNs) had returned to normal levels. Therefore, a lack of phagocytic cells could not have been responsible for the inability of CY-treated mice to survive intravenous challenge. There is a possibility, of course, which was not tested by us, that although the phagocytic cells were at normal levels at the time of challenge, they may have had altered functional capabilities. On the other hand, the spread of the organism from the site of cutaneous inoculation in CY-treated mice probably was related to a lack of phagocytic cells, since CY was administered just 3 days before the cutaneous inoculation.

The data presented here with respect to levels of peripheral blood cells and to lymphocyte functions in response to mitogens are similar to those reported in the literature and were anticipated on the basis of known effects of CY (2, 11, 18, 24, 33, 34, 37). It is quite obvious, in fact, that CY, when administered in a proper dose, affects not only lymphoid cells, primarily B-lymphocytes (23, 35) and helper and suppressor T-lymphocytes (22, 23, 25), but also other rapidly dividing cells, such as PMNs and monocytes (2, 18). It is equally clear from our own studies as well as those of others (21, 22, 37) that with the proper dose and timing, CY treatment depresses antibody formation while at the same time enhancing delayed hypersensitivity, although the reasons for enhancement may vary under different experimental conditions. In this study, we were dealing with a live microorganism as antigen, and since *Candida* cells increased in number about 100-fold in CY-treated mice, the effect of increased antigenic stimulation alone could have accounted for the enhanced response. Hurtel and Lagrange (17) also found that CY, when administered before subcutaneous inoculation of mice with viable *C. albicans*, resulted in increased delayed hypersensitivity to particulate and soluble *Candida* antigens. However, they did not evaluate their animals for acquired resistance to *C. albicans*. Without demonstrable antibody, presumably CY-treated animals, unlike normal animals, would have few primed B-cells to respond rapidly to *Candida* at the time of intravenous challenge. Antibodies have been

shown to contribute to resistance to reinfection in a thigh lesion model of candidiasis, wherein the thigh lesion was reduced in size if immune serum were administered repeatedly (28); lymphocytes from previously infected donor mice had no effect on lesion size in the same model. Additionally, Mourad and Friedman (26) were able to protect mice against systemic challenge by repeated administration of hyperimmune serum, but when the immune serum was discontinued, the mice began dying at a rate similar to that seen in animals not receiving immune serum.

In summary, the results of the present study add one more piece of evidence to support the theory that an intact cellular immune system, intact at least to the point of being capable of expressing delayed hypersensitivity or responding in vitro in a stimulation assay, is not the sole determinant of the ability to develop resistance to reinfection. The ability to form antibody quickly appears to be crucial to the survival of the animals against a systemic challenge.

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