Cyclophosphamide Effects on Murine Cryptococcosis

JOHN R. GRAYBILL[1,2]* AND LINDA MITCHELL[1]

Division of Infectious Diseases, Audie L. Murphy Memorial Veterans Hospital,[1] and the Department of Medicine, University of Texas Health Science Center,[2] San Antonio, Texas 78284

BALB/c mice were given cyclophosphamide and challenged with *Cryptococcus neoformans.* Delayed-type hypersensitivity was transiently depressed, and survival was either unaffected or shortened by cyclophosphamide.

When cyclophosphamide (CY) is administered to laboratory animals, it produces a transient decrease in peripheral blood polymorphonuclear leukocytes (PMN) and lymphocytes. Spleen and lymph node lymphocytes are also depressed. Activity of CY is selectively greater against B-lymphocytes than the thymus-dependent lymphocytes (T-lymphocytes) which effect cell-mediated immunity (8, 9). Depending on the time of immunization, cell-mediated immunity may be heightened by CY treatment. Finerty and Krehl have also associated CY pretreatment with increased protection in murine malaria (4). The present studies were undertaken in order to determine whether CY treatment alters delayed-type hypersensitivity (DTH) reactions to extracts of *Cryptococcus neoformans,* or affects the course of murine cryptococcosis.

Five-week-old male BALB/c mice were used. Some groups were immunized with cryptococcal extract emulsified in an equal volume of complete Freund adjuvant and challenged 2 weeks later (5). Other groups were immunized on the day CY was given. The test used for DTH reactivity was the mouse ear swelling test. It is analogous to the footpad swelling test, but utilizes the pinna of the ear as a test site (5). Results were measured in micrometers of antigen-specific ear swelling (SES), and were compared using one-way analysis of variance (ANOVA).

Mice were challenged intraperitoneally with our strain B *C. neoformans* (5). The 50% lethal dose within 60 days postchallenge was approximately 600 colony-forming units (CFU), with heavy cryptococcal infestation of spleen, liver, and brain at the time of death. Life table analysis was used to compare survival of various groups of mice. Either 10 days before or on the day of challenge, mice were treated subcutaneously with CY in single doses of 50, 100, or 300 mg/kg. Tail vein blood leukocyte counts were performed 1 day before, 5 days after, and 15 days after CY treatment and challenge. Controls received cryptococci but no CY, or CY but no cryptococci. Results were compared using ANOVA.

Total blood leukocytes and PMN did not change significantly in control mice challenged with cryptococci (Table 1). Whereas CY at 50 mg/kg did not affect leukocyte counts, 300 mg/kg markedly reduced both total leukocytes and PMN 5 days after administration. Counts had recovered by day 15. Both lymphocytes and PMN were affected by the higher dose of CY. CY effects on DTH are presented in Table 2. Mice infected with cryptococci on day 1 had already developed some SES reactivity to cryptococcin by day 6. Mice which received single doses of CY at 50 or 300 mg/kg had no change in SES reactivity at day 6, but were slightly larger than control mice by day 15. This delay in development of reactivity was also seen in mice which were not challenged with cryptococci, but instead immunized with cryptococcin.

Finally, CY had either no effect or a deleterious effect on survival of mice with cryptococcosis. Thirty controls received 300 mg of CY per kg but no cryptococci. Two died. Thus, drug toxicity did not account for effects on survival.

In our first study, groups of 10 mice were challenged with 10^4 *C. neoformans* and treated on the same day with either saline or 50, 100, or 300 mg of CY per kg. Survival was not altered by CY in any dose. In the next study (Fig. 1), the challenge dose was increased 10^6 CFU. At this dose, CY at 300 mg/kg was associated with a significantly increased number of deaths in the first week; thereafter, the survival curve approximated nontreated controls. When mice were pretreated with CY 10 days before challenge, their mortality was significantly higher than controls or mice treated with CY on the day of challenge (data not shown).

An additional study was conducted in mice challenged with a low dose of 10^3 CFU (Fig. 2). Immunized and nonimmunized mice were either treated with 300 mg of CY per kg or served as controls. Although CY did not affect the mor-

tality of nonimmunized mice, it ablated the prolonged survival of immunized mice. Thus, immunized mice treated with CY had similar survival to controls. A separate study was performed at a larger challenge of 10^6 CFU, with similar results (data not shown).

TABLE 1. *Tail vein blood leukocyte counts*[a]

Treatment[b]	Blood leukocyte count ($\times 10^6/cm^3$) at:		
	Day 0	Day 6	Day 16
Cr			
Total	10.9 ± 1.0^c	8.9 ± 1.5	12.2 ± 0.6
PMN	2.4 ± 0.5	1.6 ± 0.3	
Cr/CY$_{50}$			
Total	9.5 ± 1.3	7.4 ± 0.9	9.4 ± 0.8
PMN	2.1 ± 0.4	1.6 ± 0.4	
Cr/CY$_{300}$			
Total	13.7 ± 2.7	2.1 ± 0.2^d	13.0 ± 2.1
PMN	2.7 ± 0.7	0.4 ± 0.1^d	9.3 ± 0.8
CY$_{300}$			
Total	13.5 ± 1.3	4.6 ± 0.7^d	11.4 ± 1.7
PMN	3.6 ± 1.0	0.8 ± 0.5^d	6.0 ± 1.0

[a] CY and challenge given on day 1.
[b] Abbreviations: Cr, *C. neoformans* challenge, 10^6 CFU per mouse intraperitoneally; CY$_{50}$, CY$_{300}$, cyclophosphamide at a 50 or 300 mg/kg subcutaneous dose, respectively.
[c] Mean ± standard error of the mean. Each value represents the mean of at least five mice, and usually more than eight mice.
[d] Day 6 count significantly less than either 1-day count or 16-day count ($P < 0.05$ by one-way ANOVA).

The present studies included doses of CY which suppressed total leukocyte counts in peripheral blood. Like others, we could not find a dose of CY which selectively affected lymphocytes and not PMN (1, 10). However, the 50-mg/kg dose, which did not depress leukocyte counts, did delay development of SES reactivity after infection or concurrent immunization. This is consistent with CY acting against a population of T-lymphocytes stimulated by cryptococcal antigens. When DTH was tested 2 weeks after challenge, CY-treated mice had slightly larger reactions than untreated controls. However, the

TABLE 2. *SES reactivity*

Treatment[a]	SES reactivity (μm of induration) at:		
	Day 0	Day 6	Day 16
Cr	1.7 ± 0.7^b	5.0 ± 1.1^c	6.0 ± 1.1^c
Cr/CY$_{50}$	1.7 ± 1.1	3.2 ± 2.6	7.5 ± 1.1^d
Cr/CY$_{300}$	3.3 ± 0.7	3.9 ± 1.0	10.3 ± 2.4^c
CN$_{imm}$/CY$_{300}$	3.3 ± 0.7	5.2 ± 1.3	8.5 ± 1.1^c

[a] Abbreviations: Cr, *C. neoformans* challenge, 10^6 CFU intraperitoneally, given day 1; CY$_{50}$, CY$_{300}$, CY at 50 or 300 mg/kg, subcutaneous, single dose day 1; CN$_{imm}$, mice given cryptococcin immunization on day 1, not challenged.
[b] Mean ± standard error of the mean. Each value represents at least 5 mice, and usually 10 or more.
[c] Day 6 and/or day 16 SES reactivity significantly greater than day 0, one-way ANOVA.
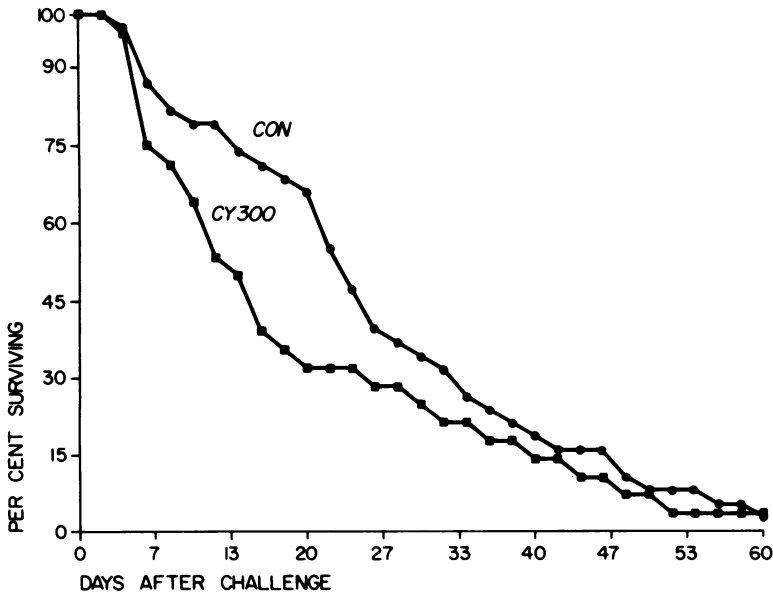[d] $P \simeq 0.07$.

FIG. 1. *Survival after challenge with 10^6 CFU of C. neoformans and treatment with CY. Abbreviations: CON, no treatment, 38 mice; CY300, CY, 300 mg/kg, 28 mice.*
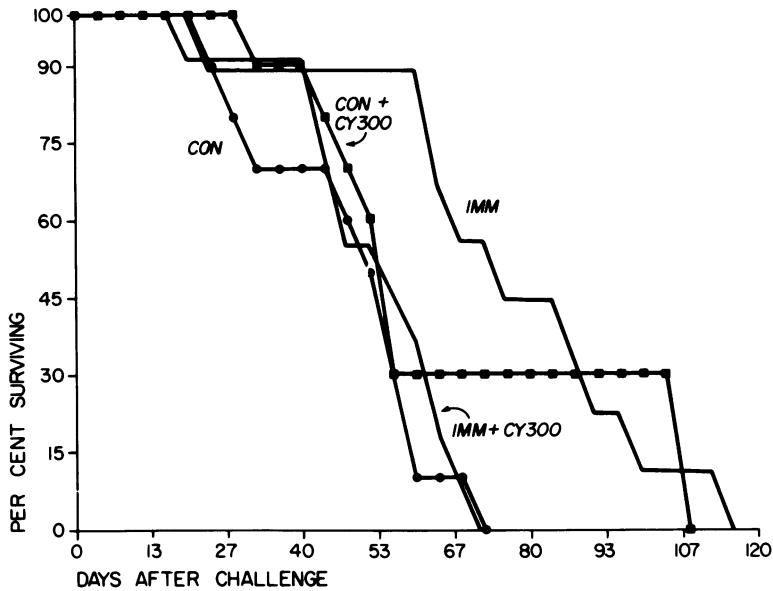
FIG. 2. *Effect of CY on previously immunized mice challenged with 10^3 CFU of C. neoformans. Abbreviations: CON, no immunization, no CY, 10 mice; CON + CY300, no immunization, 300 mg of CY per kg, 10 mice; IMM, immunized with cryptococcin, 9 mice; IMM + CY300, immunized plus CY, 300 mg/kg, 11 mice.*

differences were not statistically significant, and do not support a late "exaggerated" T-lymphocyte response as noted for murine malaria (4).

We also demonstrated no survival benefit conferred by CY, and in several studies CY appeared to increase mortality. These results do not permit conclusions as to the action of CY on a specific immune defense mechanism in cryptococcosis. There are several reasons for this. First, the only effect of CY on survival was noted at the 300-mg/kg dose, which was also associated with marked PMN depression. There is laboratory evidence suggesting that early dissemination of *C. neoformans* may be prevented by antibody-complement opsonizing phagocytosis by PMN; accordingly, acute leukopenia induced by CY might have permitted overwhelming fungal infection and early death in some mice (3). Second, in mice receiving CY 10 days before challenge, a maneuver aimed at increasing T-lymphocyte response, there was still early mortality from CY treatment (6). However, one cannot attribute this effect to low blood PMN, since PMN recovery was occurring by 10 days after CY. Finally, in mice preimmunized with cryptococcin and then given a low cryptococcal challenge, CY treatment did not affect mortality of controls but shortened the survival of immunized mice to the same range as controls. Thus, any effects of CY on host defense were negative.

CY treatment has produced a broad range of

effects in other infectious diseases. In histoplasmosis, an infection with clear dependence of host defense cell-mediated immunity, CY has adverse consequences on survival (2). In others, such as listeriosis or mouse malaria, CY appears beneficial in increasing host resistance (4, 7). These different results may be better understood after we learn whether T-lymphocytes responsible for host defenses in infections may include multiple populations with different susceptibilities to CY.

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