Correlation Between In Vivo and In Vitro Studies of Modulation of Resistance to Experimental *Candida albicans* Infection by Cyclophosphamide in Mice

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Mice receiving a single injection of cyclophosphamide (150 mg/kg) 1 to 6 days before inoculation with viable *Candida albicans* showed an increased susceptibility to the challenge accompanied by a reduction in peripheral blood polymorphonuclear leukocytes and lymphocytes as well as in spleen cellularity. Several immunological in vitro functions also appeared to be dramatically depressed. Most of these hematological and functional parameters returned to control values by day 9 after cyclophosphamide administration, at a time when resistance to *C. albicans* infection appeared to be unchanged. However, when exposure to cyclophosphamide occurred 12 to 21 days before inoculation with the live yeast, enhanced resistance was observed with the majority of the animals surviving challenge. To gain some insight into the mechanisms underlying this late increase in resistance to *C. albicans* infection after cyclophosphamide administration, we analyzed a series of immunological functions, including the in vitro candidacidal activity of polymorphonuclear neutrophils and plastic-adherent and nonadherent spleen cells as well as the activity of natural killer cells and alloreactive T lymphocytes. The results show that a numerical rebound of blood polymorphonuclear neutrophils and the appearance of a highly candidacidal cell population in the spleen may be among the factors underlying the late increase in resistance to *C. albicans* after administration of cyclophosphamide.

Immunosuppressive regimens in humans may be among those precipitating events that create an environment favorable for host invasion by *Candida albicans*, a common member of the normal microbial flora (13, 25). Cyclophosphamide (Cy), a multifunctional alkylating agent, has long been used to produce these conditions in experimental infections (1, 6, 14, 16, 18, 20). Its modulation of immune reactivity is known in such depth that the drug is regarded as a flexible means to manipulate host responsiveness to malignancies and infections in a variety of ways (4, 5, 9, 23, 39, 42, 44).

By using proper doses and appropriate timing of Cy administration, it is possible in experimental infections to selectively deplete immune functions and therefore bring into focus the relative contributions of different components of the immune system (2, 3, 15, 22, 32). In the case of *C. albicans* infections, Cy has been used to study the protective effect of immunoactive substances in severely immunodepressed mice (8, 10, 40) and to assess the role of B lymphocytes

in the acquired resistance (33). These studies showed that Cy treatment adversely affects the ability of mice to mount defensive immune reactions, a finding that is in line with the notion that Cy-treated hosts have an increased susceptibility to *C. albicans* infections (34). Following this line of research, we approached the problem of host responses to systemic *C. albicans* challenge in Cy-treated mice. The animals were studied in vivo for their ability to resist infection at different times after drug exposure, and an array of in vitro tests were devoted to clarifying the mechanisms underlying changes in susceptibility.

We found that after a single dose of Cy, an early increase in susceptibility was soon followed by a dramatic augmentation of resistance which lasted about 10 days and was also mirrored by modifications in in vitro functions.

MATERIALS AND METHODS

Mice. Inbred BALB/c Cr (*H-2^d*), C57B1/6 Cr (*H-2^b*), and hybrid (BALB/c Cr × DBA/2 Cr)F1 (CD2F1, *H-2^d/H-2^d*) mice were obtained from the Mammalian

Genetic Section, National Cancer Institute, National Institutes of Health, Bethesda, Md., and from our own colony. In the in vivo experiments, groups consisted of 8 to 10 mice. For the in vitro assays, effector cells from three to five animals were pooled.

Drugs. Cy was kindly supplied by V. L. Narayanan of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. The drug was dissolved in 0.85% NaCl sterile solution immediately before use and injected intraperitoneally in a volume of 0.1 ml/10 g of body weight.

C. albicans. The *C. albicans* strain used throughout this study was isolated from a clinical specimen and identified according to the taxonomic criteria of Lodder (26, 28). The yeast was grown 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.) until a stationary phase of growth was reached (about 24 h). Under these conditions, the culture gave a yield of approximately 2.8×10^8 cells per ml, and the organism grew as an essentially pure yeast-phase population (30). After the 24-h culture, cells were harvested by low-speed centrifugation, washed twice in saline, and diluted at the desired concentration.

Tumors. YAC-1, a tissue culture cell line of YAC, a Moloney murine leukemia virus-induced lymphoma of A/Sn origin (12), and RBL-5, a Rauscher virus-induced lymphoma of B6 origin (*H-2^b*) (17), were maintained as tissue cultures in RPMI 1640 medium (Eurobio Labs, Paris, France) supplemented with 10% fetal calf serum (FCS; GIBCO Laboratories, Grand Island, N.Y.), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Eurobio Labs, Paris, France) and 0.1% gentamicin sulfate (hereafter referred to as complete RPMI 1640).

Blood and organ culture for titration of *C. albicans* organisms. Blood samples of both Cy-treated and control mice inoculated intravenously with *C. albicans* were collected from the retroorbital plexus and hemolyzed in distilled water. The organs of mice (spleens, lungs, and kidneys) were removed aseptically and placed in a tissue homogenizer with 3 ml of 0.85% sterile NaCl solution. Each organ was homogenized and diluted with saline. The number of CFU of *C. albicans* in the specimens was determined by a plate dilution method with Sabouraud dextrose agar. The colonies were counted after a 48-h incubation at 37°C, and results are expressed as number of CFU per organ.

Leukocyte counts. Mice were bled from the retroorbital sinus. Cell counts were performed by diluting anticoagulated blood 1:20 in Türk solution and counting the cells in a corpuscle counting chamber. Differential cell counts were performed on blood smears after May-Grünwald-Giemsa staining by counting 200 leukocytes per slide.

Cell fractionation procedure: plastic adherence. A suspension of 4×10^6 effector cells in 10 ml of complete RPMI 1640 was incubated for 3 h at 37°C in a 5% CO₂ atmosphere in 93-mm petri dishes (Nunc Inter Med, Roskilde, Denmark). At the end of the incubation, the dishes were extensively washed with RPMI 1640 medium to remove the nonadherent cells. The adherent cells were recovered by scraping with a rubber policeman and then washed and suspended (viability, 80 to 90%) in complete RPMI 1640.

In vitro cytotoxicity assays. (i) Preparation of effector cells. Spleen cell suspensions were obtained by standard techniques. Peritoneal polymorphonuclear neutrophils (PMN) were induced by intraperitoneal injection of 1 ml of 10% thioglycolate medium (brewer thioglycolate medium; Difco Laboratories, Detroit, Mich.) 18 h before harvesting. The elicited cells were collected by peritoneal washing, and approximately 95% were found to be PMN by morphological criteria.

(ii) ⁵¹Cr release assay against *C. albicans*. The candidacidal activity of various effector cell populations was assessed by a previously described technique (7). Briefly, various numbers of effector cells in 0.1 ml were mixed in U-shaped, 96-well microtiter plates with 5×10^4 ⁵¹Cr-labeled *C. albicans* cells (2×10^8 cells with 300 µCi of Na₂⁵¹CrO₄ for 2 h). After a 4-h incubation at 37°C in 5% CO₂, the plates were centrifuged at $800 \times g$ for 10 min, and the radioactivity in 0.1 ml of the supernatant was measured on a γ-scintillation counter (Packard Auto Gamma 500C; Packard Instruments, Downers Grove, Ill.). The baseline ⁵¹Cr release was that of *C. albicans* incubated alone in complete RPMI 1640 medium, and in no case did it exceed 20% of total counts per minute (cpm) incorporated by target cells.

The experimental results utilized for calculation of lytic units are expressed as the percent killing in the experimental group (quadruplicate samples) above the baseline control according to the following formula: % specific ⁵¹Cr release = (cpm in experimental group – cpm spontaneous release)/(total cpm/2) × 100, where total cpm is the radioactivity incorporated by 5×10^4 *C. albicans* cells.

(iii) ⁵¹Cr release assay against Yac-1 cells. The Cr release assay has been previously described (36). Various concentrations of effector cells were incubated with 10^4 ⁵¹Cr-labeled YAC-1 target cells (5×10^6 cells with 100 µCi of Na₂⁵¹CrO₄ for 45 min) for 4 h at 37°C in U-shaped, 96-well plastic microtiter plates (Greiner Laboratechnik, Nürtingen, Germany). After incubation, the plates were centrifuged at $800 \times g$ for 10 min, and the radioactivity in 0.1 ml of the supernatant was measured on a γ-scintillation counter. All groups were tested in quadruplicate. Baseline ⁵¹Cr release was determined by the use of an autologous control with equal numbers of unlabeled target cells in the place of effector cells. The percentage of specific lysis utilized for the calculation of lytic units was obtained as follows: % cytotoxicity = (test cpm – autologous cpm)/(total cpm incorporated/2) × 100, where test cpm is the mean cpm released in the presence of effector cells.

(iv) Generation of cytotoxic T lymphocytes in vitro. Spleen cells (responder cells, 35×10^6) from normal or Cy-treated BALB/c mice (*H-2^d*) were cocultured in vitro with irradiated (5,000 rads in vitro delivered by a ⁶⁰Co irradiator [Hot Spot; MKIV, United Kingdom Atomic Energy Authority, Harwell, England]) allogeneic spleen cells from C57B6 mice (*H-2^b*) (sensitizer cells; responder/sensitizer cell ratio, 8:1) in 30-ml tissue culture flasks (Costar, Cambridge, Mass.) in a final volume of 20 ml, the cells were suspended in RPMI 1640 medium buffered at pH 7.2 with sodium bicarbonate and supplemented with 15% heat-inactivated fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES buffer, and 50 µg of gentamicin per ml as previously described (21). The

cultures were incubated at 37°C in a 5% CO₂ incubator for 5 days.

(v) ⁵¹Cr-release assay against RBL-5. At the end of the in vitro sensitization, the lymphoid cells were harvested, washed, and suspended in RPMI 1640 containing 20% fetal calf serum and 10 mM HEPES buffer and used as attacker cells in a ⁵¹Cr release microcytotoxicity assay against ⁵¹Cr-labeled RBL-5 lymphoma cells as described above for Yac-1 cells.

Calculation of cytotoxic and lytic units. Dose-response curves were obtained by plotting the percentage of specific ⁵¹Cr release and the attacker/target ratio (3 to 5 for each curve) for all cytotoxic activities. The best-fit curve for these functions was found to be logarithmic (Hewlett-Packard calculator H-p 97, program standard Pac, 03-01) in accordance with previous reports (11, 29, 43). For candidacidal or natural killer (NK) activities, a cytotoxic unit (CU₂₀) or a lytic unit (LU₂₀), respectively, was defined as that number of attacker cells, extrapolated from the dose-response curve, that was required to achieve 20% specific target cell killing. The amount of CU₂₀ per spleen or LU₂₀ per spleen was calculated by dividing the total number of spleen cells by the number of splenocytes corresponding to CU₂₀ or LU₂₀.

Statistical analysis. Differences in survival times were analyzed by the Mann-Whitney U test. Differences in CFU in the various organs, in the number of circulating peripheral leukocytes, and in the number of CU or LU in the in vitro microcytotoxicity assays were determined according to Student's *t* test. Each experiment was repeated three to five times.

RESULTS

Effect of time of Cy administration on in vivo resistance against *C. albicans*. To assess a kinetic pattern of Cy-induced modifications in susceptibility to *C. albicans* infection, we studied the effect of drug treatment performed at different times before systemic challenge with the yeast.

Male CD2F1 mice were treated with the drug 6 h or a number of days (3, 6, 9, 12, 15, 18, 21, or 24 days) before the intravenous challenge with 5×10^5 live yeast cells. Figure 1 shows that treatment with Cy 3 or 6 days before challenge significantly increased susceptibility to infection as shown by the reduction in median survival time of the challenged mice (Fig. 1B). No long-term survivors, i.e., animals surviving the 60-day observation period, were found among these animals (Fig. 1A). On the contrary, exposure to Cy 12, 15, 18, or 21 days before *C. albicans* injection significantly augmented the resistance of the treated mice, as shown by the presence of long-term survivors (Fig. 1A) and increased median survival times (Fig. 1B). Treatment performed 12 days before *C. albicans* challenge was the most effective in enhancing resistance to infection. It appears, therefore, that pretreatment with Cy (150 mg/kg) can either depress (on day 3) or augment (on day 12, 15, 18, or 21) resistance to subsequent *C. albicans* infection.

FIG. 1. Effect of the time of Cy administration on survival of CD2F1 mice challenged intravenously with 5×10^5 *C. albicans* cells. Cy (150 mg/kg) was given intraperitoneally as a single injection at different times before *C. albicans* challenge. Symbols: C, Untreated control mice; ↓, Cy administered 6 h before *C. albicans*; * $P < 0.01$ (Cy-treated mice versus controls).

Dose dependency of depressive and enhancing effects on *C. albicans* resistance after Cy treatment. To further analyze the effects of Cy on *C. albicans* resistance, experiments were performed in which a wide range of drug doses were administered at different times before *C. albicans* challenge. Figure 2 shows the results of an experiment in which 54, 90, 150, or 250 mg of Cy per kg was administered 3 (Fig. 2A) or 12 (Fig. 2B) days before challenge.

It is apparent that both the depressive and enhancing effects were dose dependent; no significant modification in susceptibility to *C. albicans* infection was observed when Cy was used at the dose of 54 mg/kg. At the higher doses tested, the depressive effect was always present, whereas enhancement of resistance had a peak around 150 mg/kg.

Effect of Cy treatment on the recovery of live *C. albicans* from different organs. A subsequent series of experiments was devoted to establishing whether modifications in susceptibility to *C. albicans* challenge could be directly related to the in vivo killing of the injected yeast particles. To this purpose, animals given Cy 3 or 12 days earlier were then injected with 5×10^5 live *C. albicans* cells, and the survival of the microorganisms in different organs (kidneys, lungs, spleen,

FIG. 2. Effect of different doses of Cy on the survival of CD2F1 mice challenged intravenously with 5×10^5 *C. albicans* cells. (A) Cy was given 3 days before challenge; (B) Cy was given 12 days before challenge; (C) untreated controls. Symbol: *, $P < 0.01$ (Cy-treated mice versus controls).

and blood) was assessed at different times in terms of CFU. Figure 3 shows that, as early as 6 to 8 h after *C. albicans* injection, the percent recovery of live microorganisms varied considerably in relationship to the treatment in all of the tested organs.

Blood clearance or killing of the yeast appeared severely impaired in mice exposed to Cy 3 days earlier, whereas the percent recovery in the organs of mice treated with the drug 12 days before was significantly lower than that found in untreated controls, thus suggesting that an enhanced killing of the injected microorganism had occurred. The differences were most marked in the lungs and spleen at about 8 to 24 h after *C. albicans* injection.

Effect of Cy treatment on hematological and lymphoid parameters. It is known that resistance to *C. albicans* infection relies on contributions by a variety of cellular effectors of the immune system. It was therefore of interest in our study to search for a relationship between modifications in *C. albicans* resistance after exposure to Cy and number of peripheral blood leukocytes and spleen cells. In line with the data in the literature (33, 38), we found that after administration of Cy (150 mg/kg) there were indeed remarkable variations in the actual number of leukocytes in the blood. An early (3 to 6 days)

decrease in total leukocyte counts, largely resulting from a drop in lymphocyte and granulocyte counts, was soon followed by a dramatic increase in total leukocyte counts by days 9 through 12, which was mostly accounted for by an increase in granulocyte number detectable up 15 days after Cy administration (data not shown). When the examination was extended to the spleen (Fig. 4), we found that an early drop in weight by days 3 through 6 was accompanied by a considerable increase at 9 to 15 days after treatment (Fig. 4A). This was reflected by variations in spleen cellularity (Fig. 4B); after an initial decrease at 3 days, a remarkable augmentation was observed at about 12 days, which was mostly accounted for by the expansion of splenocytes not adhering to the plastic surface.

In vitro candidacidal activity of granulocytes after Cy treatment. The results so far reported suggested that modifications in in vivo susceptibility to *C. albicans* infection after exposure to Cy could be related, at least in part, to modifications in number or functional activity (or both) of blood granulocytes as well as nonadherent spleen cells. Experiments were therefore designed to monitor the in vitro candidacidal activity of these populations. We have recently shown that a reliable method for quantitating the in vitro killing of *C. albicans* is the assessment of radiolabel release from ^{51}Cr -labeled yeast particles incubated with mouse effector cells for 4 h (7).

In experiments employing mice, the source of granulocytes is represented by the peritoneal cavity of animals undergoing a sterile peritonitis (41) caused by the injection of thioglycolate broth. Figure 5 shows the cytotoxic ability of peritoneal thioglycolate-induced granulocytes against *C. albicans* measured at different times after the injection of Cy (150 mg/kg). Results are expressed as $\text{CU}_{20}/10^6$ cells as described above.

It is apparent that exposure to Cy resulted in an immediate loss of activity with a negative peak at 3 days of treatment. At about 10 days, however, the candidacidal activity had returned to control values. In all exudates, the percent PMN as assessed by morphological criteria was in the 85 to 95% range, the only exceptions being Cy on day -3 (percent PMN, 24%) and Cy on day 0 (percent PMN, 63%).

In vitro candidacidal activity of spleen cells after Cy treatment. A series of experiments similar to those performed with granulocytes was also run with splenic effectors. Total spleen cells and the plastic adherent and nonadherent fractions were reacted in vitro against ^{51}Cr -labeled *C. albicans* particles at different times after the injection of Cy. Figure 6 shows the results in terms of both $\text{CU}_{20}/10^6$ cells (Fig. 6A)

FIG. 3. Effect of Cy treatment on the recovery of live *C. albicans* particles from untreated mice (■), from mice given Cy (150 mg/kg) 3 days before the assay (○), and from mice given Cy 12 days before the assay (△). CD2F1 mice were injected with the microorganism, and then their organs were processed at different times for assessment of survival and multiplication of the yeast. Results are expressed as percent recovery, calculated on the basis of the obtained CFU. Symbols: ↓, 5 min after *C. albicans* inoculation; *, $P < 0.01$ (Cy-treated mice versus controls).

and CU_{20} per spleen (Fig. 6B). It appears that on day 3 of drug administration, all of the tested populations underwent a dramatic loss in reactivity that was followed by complete recovery (adherent fraction) or even marked augmentation (unfractionated and nonadherent spleen cells) of the candidacidal potential during the subsequent 15 days (Fig. 6A). These differences were magnified when the results were expressed as CU_{20} per spleen, which takes into account the previously described increase in cellularity occurring at about 9 days after Cy administration (Fig. 6B). It is apparent that the candidacidal potential of the splenic nonadherent fraction remarkably increased by day 12 after Cy treatment.

Effect of Cy on the generation of CTL in vitro. Studies were performed to evaluate the effect of Cy treatment on T-dependent immune functions such as the in vitro generation of alloreactive cytotoxic T lymphocytes (CTL). BALB/c (*H-2^d*) mice treated with Cy (150 mg/kg) at different

times were then used as a source of splenic lymphocytes to be cocultured with stimulating spleen cells from C57B1/6 animals (*H-2^b*). After a 5-day culture the cytolytic activity of the responder lymphocytes was tested against ^{51}Cr -labeled RBL-5 (*H-2^b*) lymphoma cells in a 4-h ^{51}Cr release assay. The results we obtained provided evidence that exposure to Cy 3 to 12 days before the allogeneic coculture significantly impaired the ability of the responder lymphocytes to generate cytotoxic effectors (data not shown).

Effect of Cy treatment on NK cell activity. The relationship between the activity of NK cells and modulation of immune reactivity by Cy was investigated in 6- to 8-week-old CD2F1 mice at different times (day 3, 6, 9, 12, 18, and 21) after the injection of the drug (Fig. 7).

The cytotoxic activity of splenic NK effectors was measured in a 4-h ^{51}Cr release assay against labeled YAC-1 lymphoma cells at different effector/target ratios. Results are expressed both in

FIG. 4. Effect of the time of Cy administration on weight (A) and cellularity (B) of the spleen of CD2F1 mice. Symbols: C, control mice; ●, unfractionated spleen cells; ■, plastic adherent spleen cells; ▲, nonadherent spleen cells; *, $P < 0.01$ (Cy-treated mice versus controls). Cy (150 mg/kg) was given as a single injection on day 0.

terms $LU_{20}/10^6$ cells and LU_{20} per spleen. It is obvious that after an early and dramatic drop in reactivity a considerable augmentation of the cytotoxic potential occurred starting from day 6 up to day 18. Both the activity of individual effectors (Fig. 7A) and the total lytic potential of the spleen (Fig. 7B) underwent considerable augmentation.

DISCUSSION

In the present paper we report on a series of studies aimed at assessing possible links between in vivo resistance of Cy treated mice to *C. albicans* challenge and in vitro functional activity of different components of the immune system. As expected and in line with previous results (20), susceptibility to *C. albicans* appeared to be increased shortly (3 to 6 days) after Cy exposure (Fig. 1), a finding that could be easily anticipated on the basis of the known effects of Cy. Indeed, this agent, given as a single dose of 100 to 200 mg/kg, affects most lymphoid cells, primarily B lymphocytes (4), helper and suppressor T lymphocytes (27, 31, 37, 45), and rapidly dividing cells such as PMN and monocytes (20).

Furthermore, it has remarkable effects on the in vitro functional activity of immune cytotoxic effectors (19). It was not surprising, therefore, to find that the increase in susceptibility to *C. albicans* infections was accompanied by an immediate depression of peripheral blood leukocytes and a fall in spleen cellularity. The in vitro candidacidal activity of PMN and spleen cells (both the plastic adherent and nonadherent fractions) was depressed (Fig. 5, Fig. 6).

On day 9 after Cy administration the in vivo resistance to *C. albicans* challenge appeared unaffected (Fig. 1) at a time when PMN and spleen lymphocytes were rebounding to normal or above-normal levels. On days 12 through 21

FIG. 5. Candidacidal activity of granulocytes from Cy-treated mice. CD2F1 mice exposed to Cy (150 mg/kg) a number of days earlier served as a source of PMN to be reacted against ⁵¹Cr-labeled *C. albicans*. At 18 h before testing, PMN in the Cy-treated mice were elicited by injection of thioglycolate. Symbols: ↓, Cy given 6 h before testing and therefore 12 h after thioglycolate; C, untreated controls; *, $P < 0.01$ (Cy-treated mice versus controls).

FIG. 6. Candidacidal activity of spleen from Cy-treated mice. CD2F1 mice treated with Cy (150 mg/kg) at different times were donors of spleen cells to be reacted against radiolabeled *C. albicans* particles. Results are expressed as $CU_{20}/10^6$ cells (A) and CU_{20} per spleen (B). Symbols: ●, unfractionated splenocyte; ▲, plastic-adherent splenocytes; ■, nonadherent splenocytes; C, untreated controls; *, $P < 0.01$ (Cy-treated mice versus controls).

after Cy treatment enhanced resistance was observed (Fig. 1) which was paralleled by maximal increase in PMN and spleen cellularity. The in vitro candidacidal activity of PMN and nonadherent spleen cells had returned to normal or above-normal levels. The greater resistance of animals treated with Cy 12 days earlier as compared with controls or mice receiving the drug 3 days before microbial challenge was presumably due to an enhanced clearance of the yeast as shown by the percent recovery (in CFU) of *C. albicans* in different organs. Differences among the groups were most obvious at 8 to 24 h after challenge in the spleen and lungs, organs which act as blood filters and represent sites of active immunological cytotoxic reactions.

Thus, it appeared that both the depressive and enhancing effects of Cy on resistance to *C. albicans* infection were related to the in vivo ability of the host to counteract the systemic spread of the intravenously injected yeast. This activity could reasonably be mediated by those cell populations recovering (as suggested by previous results [20]) or rebounding in concurrence with the increase in resistance. We have already mentioned that, by day 12 of Cy administration, the number of peripheral blood PMN was far above normal levels, whereas their indi-

vidual activity as candidacidal effectors was unchanged. Thus, the overall killing potential of blood PMN was presumably much greater. Even more noteworthy was the reactivity of spleen cells, which showed both an increase in the total number and an augmentation of activity in plastic nonadherent effectors. This resulted in a dramatic potentiation of the capability of killing against the infectious microorganism. These plastic nonadherent splenocytes were not phagocytic as measured by their ability to ingest *C. albicans* particles (data not shown) or nylon-nonadherent particles (data not shown), nor did they bear detectable amounts of Thy 1.2 antigen (as proved by exposure to anti Thy 1.2 serum plus complement, data not shown). On the other hand, the in vitro generation of cytotoxic T lymphocytes showed that T lymphocyte-dependent responses were severely compromised up to 18 days after treatment of mice with 150 mg/kg, a result that is in line with previous data in the literature (37).

The recent interest in natural cell-mediated cytotoxicity in vitro against microorganisms (24, 35) and its links with the expression of NK cell activity against tumors (35) led us to examine the reactivity of the subpopulation of natural cytotoxic effectors capable of killing ^{51}Cr -labeled

FIG. 7. Effect of Cy treatment on NK cell activity. CD2F1 mice given Cy a number of days earlier were tested for their NK cell lytic potential. Results were expressed as LU₂₀/10⁶ cells (A) and LU₂₀ per spleen (B). Symbols: C, untreated controls; *, $P < 0.01$ (Cy-treated mice versus controls).

YAC-1 targets in a 4-h assay with Cy-treated mice as donors of splenic lymphocytes. It was found that, after an early (day 3) drop in reactivity, the levels of NK expression increased to above normal values starting from days 6 through 9 after Cy treatment and up to day 18. Taken together, the data obtained in the present study show that the in vivo resistance of the mice to challenge with *C. albicans* can be either decreased or enhanced by Cy depending on the time of drug administration. The effect is also dose dependent, and the optimal range for protection is 90 to 150 mg/kg. Although it is plausible that Cy doses higher than 150 mg/kg may result in nonselective toxicity to the host, thus nullifying any beneficial anti-*C. albicans* effect, it is not yet clear why doses lower than 90 mg/kg fail to confer protection. Experiments are under way to clarify the issue.

An early (3 to 6 days) drop in peripheral blood leukocyte count and spleen cellularity associated with a decreased candidacidal activity in vitro may be among the factors underlying the impaired clearance of the intravenously injected yeast. The late (12 to 21 days) augmentation of resistance is concurrent, on the other hand, with

a numerical rebound on PMN in the blood, the individual activity of which is apparently normal. Moreover, the spleen becomes the site of an enriched population of "activated" candidacidal effector cells lacking the characteristics of mature T cells and macrophages, which will be further characterized in a subsequent study (Baccarini et al., manuscript in preparation). Concurrent with the increase in resistance in vivo is the activation of a subpopulation of natural cytotoxic effectors reactive in vitro against YAC-1 tumor cells.

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