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In Vivo Immunosuppressive Activity of Gliotoxin, a Metabolite Produced by Human Pathogenic Fungi

P. SUTTON,* N. R. NEWCOMBE, P. WARING, AND A. MÜLLBACHER

Division of Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory 2601, Australia

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Aspergillosis is a disease caused by the opportunistic pathogen *Aspergillus fumigatus* and other related fungi. It occurs mainly in immunosuppressed people and causes very high mortality rates. *A. fumigatus* and other pathogenic fungi have been shown to produce a metabolite, gliotoxin, which has immunosuppressive properties in vitro, but little is known about its in vivo activity. Here we report that gliotoxin has increased toxicity in mice after irradiation. A single injection of gliotoxin delayed the recovery of immune cells after immunosuppression by sublethal irradiation by 2 weeks. Study of the morphology of cells of the thymus, spleen, and mesenteric lymph nodes by light microscopy and electron microscopy and agarose gel electrophoresis of DNA from these organs showed that the injection of gliotoxin induced apoptosis in cells of the immune system in vivo. Thus, gliotoxin does have immunosuppressive activity in vivo and could potentially play a significant role in the pathogenesis of aspergillosis and other fungal diseases.

Aspergillosis is a very serious opportunistic infection which can occur in immunosuppressed patients, such as cancer sufferers who undergo irradiation and/or chemotherapy treatment (1a), sufferers of immune deficiency diseases such as AIDS (14), and organ transplant recipients who are given immunosuppressive drugs to prevent graft rejection (7, 19). Despite the development of new antifungal agents such as itraconazole (4) and the use of the immunosuppressive drug FK 506, which may lower the incidence of *Aspergillus* infections in transplant patients (18), mortality due to such infections is still very high. Among infections caused by pathogenic fungi, the incidence of aspergillosis is second only to that of *Candida* infections, and aspergillosis produces the greater mortality.

Aspergillosis is most commonly caused by the fungus *Aspergillus fumigatus*. *A. fumigatus*, as well as other fungi including some *Candida*, *Penicillium*, and *Gliocladium* species (3, 15, 17), produces the secondary fungal metabolite gliotoxin (16). Gliotoxin belongs to the epipolythiodioxopiperazine family and has been shown to possess in vitro immunosuppressive activity (8, 9). As yet no direct link has been established between the production of this metabolite and the pathogenesis of aspergillosis, and all evidence for the immunosuppressive properties of gliotoxin has so far come from in vitro studies. However, gliotoxin has been isolated from mice undergoing systemic experimental aspergillosis (6). Gliotoxin has been shown to inhibit phagocytosis by activated peritoneal macrophages (5, 8) and also to inhibit mitogen-induced T-cell proliferation (12) and the activation of cytolytic T lymphocytes, partially by inhibiting lymphokine release (8). The ex vivo treatment of bone marrow with gliotoxin prevents graft-versus-host disease in allogeneic bone marrow transplantation by selectively depleting the bone marrow of mature lymphocytes (11).

Thus, gliotoxin, produced in the vertebrate host, could play a significant role in the pathogenesis and mortality of aspergillosis. The potential importance of gliotoxin is indicated by the observation that it is synthesized by many different fungi, including strains of *Candida* (15).

* Corresponding author.

Here we present data showing that gliotoxin has increased toxicity in irradiated mice, that it delays the recovery of the immune system in vivo after immunosuppression by sublethal irradiation, and that it induces apoptosis in the cells of lymphoid organs. We thus demonstrate that gliotoxin does have immunosuppressive activity in vivo, which could have significant implications for mortality caused by infections with gliotoxin-producing fungi.

MATERIALS AND METHODS

Mice. Female CBA/H mice between 6 and 8 weeks old were used in these experiments. They were bred pathogen free in the John Curtin School of Medical Research animal unit, Canberra, Australia.

Gliotoxin. Gliotoxin was prepared from cultures of *Penicillium bilaii* by methods previously described (21) and was stored in powder form at −70°C. Radiolabelled gliotoxin was prepared as cited above except that the fungus was incubated with 5 mCi of $Na^{35}SO_4$ (Amersham) in 150 ml of culture medium. Immediately prior to injection, gliotoxin was initially dissolved in dimethyl sulfoxide at 2 mg/ml and diluted to 100 μg/ml in warmed phosphate-buffered saline (PBS). Appropriate volumes of solution were then intraperitoneally injected. Control animals received 2 ml of a 5% dimethyl sulfoxide solution intraperitoneally.

Irradiation. Mice were irradiated by a ^{60}Co source.

Proliferation assays. Splenocytes were erythrocyte depleted by hypotonic lysis, and the remaining cells were counted with a hemocytometer. To measure the cellular response of splenocytes to mitogens, 2×10^5 cells per well were aliquoted into 96-well plates in 200 μl of F15 culture medium (Multicel) supplemented with 10% (vol/vol) fetal calf serum (CSL, Parkville, Australia) and 5×10^{-5} M 2-mercaptoethanol, with or without concanavalin A (ConA) (4 μg/ml) and lipopolysaccharide (LPS) (10 μg/ml). After 2 days in a humidified incubator at 37°C, each well was pulsed for 6 h with 1 μCi of tritiated thymidine (Amersham) and the counts were measured in a scintillation counter (1205 betaplate, Pharmacia).

Fluorescence-activated cell sorter (FACS) analysis. All procedures were performed at 4°C. Splenocytes were resuspended

TABLE 1. Toxicity of gliotoxin on sublethally irradiated mice[a]

Level of irradiation (Gy)	No. of mice that died/no. of mice injected with[b]:			
	50 μg	100 μg	150 μg	200 μg
0	ND	0/6	3/15 (2.3)	4/5 (1.5)
4	ND	0/20	8/22 (4.0)	ND
6	0/15	15/60 (3.2)	8/11 (2.5)	3/4 (2)

[a] The mean body weight was 17.7 ± 0.8 g.
[b] Numbers in parentheses are mean survival time, in days, of animals that died. ND, not done. Gliotoxin was injected intraperitoneally.

in PBS plus 2% (vol/vol) fetal calf serum at 10^7 cells per ml. One hundred-microliter aliquots of cells were incubated with or without 50 μl of antibody for 30 min on ice and were washed twice with buffer. A concentrated cell culture supernatant of the rat antibody RA3-6B2 (a gift of P. Hodgkin, John Curtin School of Medical Research), which binds to the mouse B-lymphocyte high-molecular-weight form of CD45, was used. Antibody binding was detected by incubating a further 30 min with a mouse anti-rat kappa-chain antibody, conjugated to fluorescein isothiocyanate. Samples were washed twice more and analyzed by using a Becton Dickinson FACScan.

Histology. For light microscopy, tissue samples were fixed in 10% neutral buffered formalin for at least 3 days before being embedded in wax, sectioned, and stained with hematoxylin and eosin.

For electron microscopy, samples were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4), stained with lead citrate and uranyl acetate, and analyzed with a Philips 301 electron microscope.

DNA gel electrophoresis. DNA was extracted from cells by the phenol-chloroform method, electrophoresed on 1.5% agarose gels containing 50 μg of ethidium bromide per ml, and photographed under UV light as previously described (20).

RESULTS

Gliotoxin toxicity in vivo. Fungi such as *A. fumigatus* do not cause overt disease in the immunocompetent host. In order to investigate the activity of gliotoxin in vivo, an immunosuppressed mouse model was used. Sublethal irradiation causes transient immunosuppression in mice. We asked whether gliotoxin could delay the recovery of immunocompetence in mice after irradiation. To determine a suitable dose of gliotoxin, mice were intraperitoneally injected with gliotoxin 5 days after receiving either 4 Gy, 6 Gy, or no irradiation. The results, shown in Table 1, demonstrate that without irradiation, a dose of >100 μg per mouse was required to produce lethal effects. However, if radiation treatment was given, an inverse relationship between the dose of radiation received and the amount of gliotoxin required to cause the death of the animals was apparent. These data approximated 50% lethal dose (LD_{50}) values of 9.9 and 7.9 mg/kg after 0 and 6 Gy, respectively. No LD_{50} value is given for 4 Gy, as only one point was obtained. On the basis of these data a dose of 100 μg of gliotoxin was chosen as suitable, as this dose would allow a sufficient number of animals to survive for the experimental protocol to be executed while maximizing our chance to observe an effect of gliotoxin on immune recovery.

The immunosuppressive activity of gliotoxin in vivo. To determine the effect of gliotoxin on the recovery of the immune system in mice after sublethal irradiation, mice were exposed to 4 or 6 Gy and 5 days later received 0 or 100 μg of gliotoxin intraperitoneally. Twice weekly, a mouse was sacrificed from each of these groups and from an age-matched, unirradiated control group, and their spleens were removed. The number of leukocytes in the spleens was counted, and the proliferative responses of these cells to ConA and LPS were measured. Gliotoxin injected into mice which had been exposed to 4 Gy had affected neither leukocyte numbers nor the proliferative responses compared with those in the untreated controls (data not shown). However, the injection of 100 μg of gliotoxin into mice which had received 6 Gy produced a reduction in the numbers of leukocytes present in the spleens compared with the numbers in irradiated controls (Fig. 1a). The T-cell response in irradiated animals was depressed for at least 40 days postirradiation, and the difference between gliotoxin-treated and untreated irradiated mice was small (Fig. 1b). The apparent increase occurring on day 34 was caused by an unusually low control value and so is probably not significant. However, when the proliferative response to LPS of mature B cells was measured, gliotoxin caused a marked delay in the recovery of the B-cell response (Fig. 1c).

FACS analysis of B-lymphocyte populations in the spleen after gliotoxin treatment. The delay in recovery of LPS-responsive cells in the spleens of irradiated mice may have been due to a reduction in the number of mature B cells by gliotoxin. In the proliferation assays, numbers of leukocytes were normalized, so differences in response may be due to variations in the percentages of lymphocyte subpopulations present in the spleens. Splenocytes of irradiated mice, with and

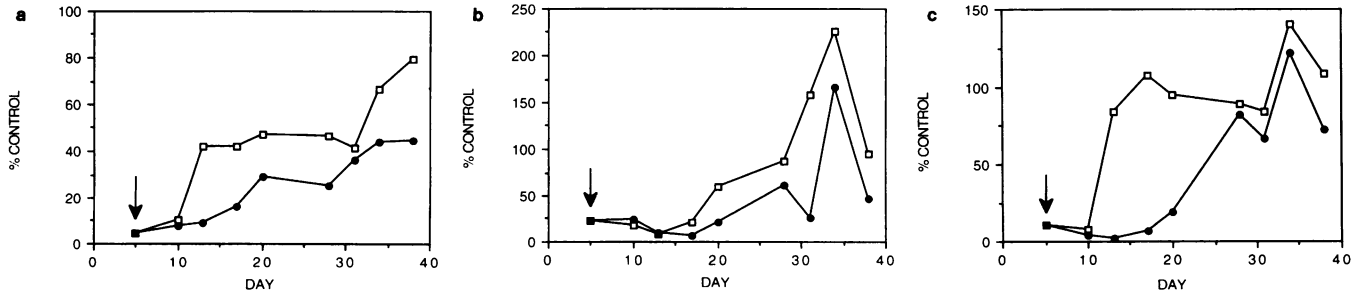

FIG. 1. Effect of gliotoxin on the recovery of splenic lymphocyte populations in irradiated mice. Data were acquired from two experiments and show the total number of leukocytes in the spleen (a) and their proliferative response to ConA (b) and LPS (c). For clarity, data are expressed as a mean percentage of age-matched, unirradiated controls. Mice were irradiated with 6 Gy on day 0. Gliotoxin treatment produced a significant reduction in the numbers of leukocytes present in the spleens and in their responses to LPS as shown by the Wilcoxon signed ranks test (P = 0.0625). There was no significant difference in the ConA responses of these cells. □, untreated mice; ●, mice injected with 100 μg of gliotoxin. Arrows indicate the time at which gliotoxin was injected.

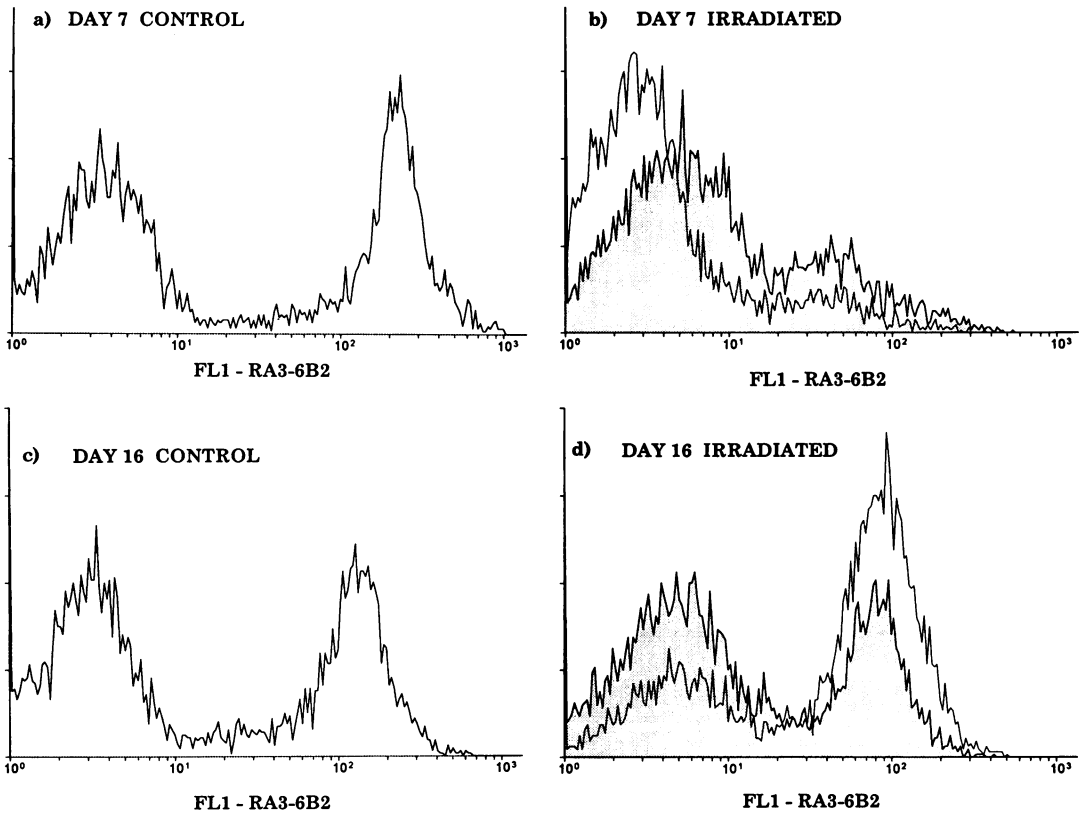

FIG. 2. Quantitation using FACS analysis of splenic B lymphocyte populations after gliotoxin treatment. Erythrocyte-depleted splenocytes were stained with RA3-6B2, an antibody against murine B cells. The *y* axis represents cell frequency. (a and c) Populations of control spleens from untreated mice. (b and d) Populations of spleens from irradiated mice; shaded profiles are those of spleens from irradiated mice injected with gliotoxin on day 5 and nonshaded profiles are those of spleens from mice which did not receive gliotoxin.

without gliotoxin treatment, were stained with an anti-B-cell antibody (RA3-6B2) and analyzed by FACS to measure the percentage of B lymphocytes present in the mice's respective spleen populations. The FACS profiles obtained (Fig. 2) show that 7 days after irradiation, prior to the recovery of normal cell numbers in the spleens and at a time when very little B-cell proliferation occurs, there was no reduction in the proportion of B cells in gliotoxin-treated mice compared with the proportion in untreated mice (Fig. 2b). A significant difference was noted 16 days after irradiation: mice which had received gliotoxin had a greatly reduced proportion of B lymphocytes (37%) in comparison with that in untreated mice (70%). This observation was made at the same time that low LPS responsiveness was observed in splenocytes of gliotoxin-treated animals; the reduction was thus due to low responder numbers.

Gliotoxin causes cells to become apoptotic in vivo. As gliotoxin had been shown to specifically affect cells of the immune system in vivo, it was important to determine how the toxin exerted its effect. Cells treated with gliotoxin in culture have been shown to undergo "programmed cell death," or apoptosis. In order to look for evidence of apoptosis in the lymphoid organs of mice, the thymuses, spleens, and mesenteric lymph nodes of unirradiated CBA mice were removed, 6 and 24 h after intraperitoneal injections of 0, 100, or 200 μg of gliotoxin. The organs were cut into thirds and studied for evidence of apoptosis by three methods: light microscopy, electron microscopy, and agarose gel electrophoresis of extracted DNA.

The appearance of spleen tissue under the light microscope after the injection of gliotoxin is shown in Fig. 3. The injection of 200 μg of gliotoxin, a lethal dose (Table 1), led to the death of a large proportion of cells in the spleen (Fig. 3e). The morphology of these cells, small with densely stained nuclei, is indicative of cells dying by apoptosis. The injection of 100 μg of gliotoxin, a sublethal dose, produced a large increase in the number of apoptotic cells in the spleen after 6 h (Fig. 3a and b). No apoptotic cells were apparent after 24 h, although cells in both organs appeared less densely packed, suggesting some cell depletion (Fig. 3c). Similar effects were observed in the thymus and mesenteric lymph nodes (not shown).

The study of these same organs by electron microscopy confirmed these observations. There was little evidence of apoptosis in splenocytes of control organs (Fig. 4a). However, after the injection of gliotoxin, apoptotic cells could be seen within 6 h with both 100- and 200-μg doses (Fig. 4b and d). Very few individual apoptotic cells could be seen after 24 h in mice that had received 100 μg of gliotoxin. At this time point, macrophages within the spleen contained many small dense bodies (Fig. 4c). This suggests that the apoptotic cells seen after 6 h might have been cleared by phagocytes. There were still a significant number of apparently undamaged cells present (Fig. 4c). Twenty-four hours after the injection of 200 μg of gliotoxin, the majority of splenocytes were apoptotic (Fig. 4e). Similar results were obtained with sections of thymus and mesenteric lymph nodes (not shown).

DNA extracted from cells of these organs was electrophoresed on agarose gels to look for DNA laddering, which is characteristic of cells undergoing apoptosis. The gel shown in

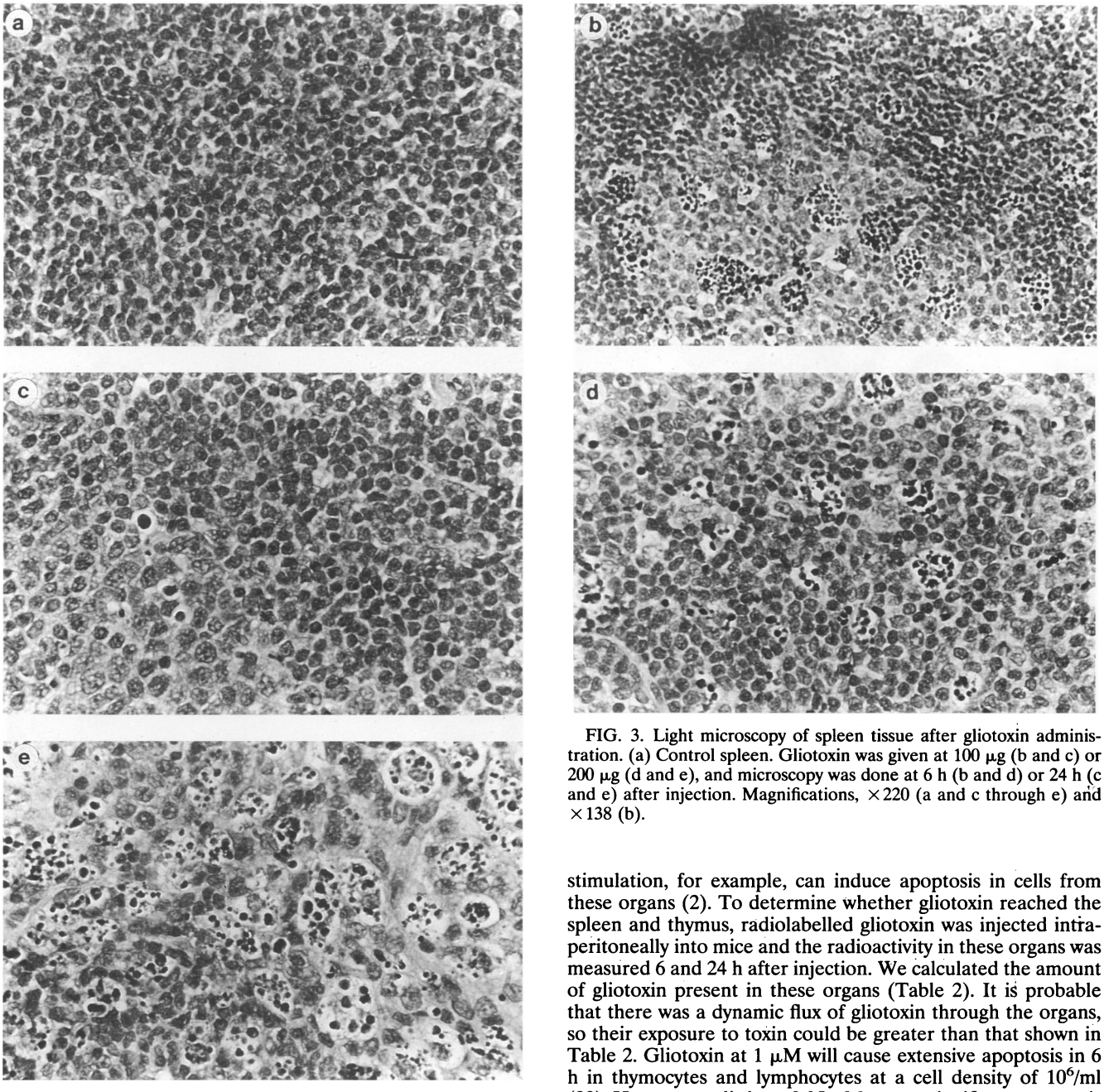

FIG. 3. Light microscopy of spleen tissue after gliotoxin administration. (a) Control spleen. Gliotoxin was given at 100 μg (b and c) or 200 μg (d and e), and microscopy was done at 6 h (b and d) or 24 h (c and e) after injection. Magnifications, ×220 (a and c through e) and ×138 (b).

Fig. 5 demonstrates the presence of DNA fragmentation in lymphoid organs of mice injected with 200 μg of gliotoxin. The highest fragmentation occurred in the thymus and was greater at 24 h than at 6 h postinjection. Fainter bands were also visible in the spleen and lymph node samples. No fragmentation could be seen in organs of mice receiving only 100 μg of gliotoxin. Although histology suggested that some apoptosis was occurring, this may not have been sufficient to be detected by agarose gel electrophoresis.

Presence of gliotoxin in lymphoid organs. It was not known if the production of apoptosis by gliotoxin in lymphoid organs was due to a direct or indirect effect. Both corticosteroid release, which occurs during stress, and loss of cytokine stimulation, for example, can induce apoptosis in cells from these organs (2). To determine whether gliotoxin reached the spleen and thymus, radiolabelled gliotoxin was injected intraperitoneally into mice and the radioactivity in these organs was measured 6 and 24 h after injection. We calculated the amount of gliotoxin present in these organs (Table 2). It is probable that there was a dynamic flux of gliotoxin through the organs, so their exposure to toxin could be greater than that shown in Table 2. Gliotoxin at 1 μM will cause extensive apoptosis in 6 h in thymocytes and lymphocytes at a cell density of 10^6/ml (20). However, as little as 0.05 μM causes significant apoptosis in thymocytes within 6 h (1). With the spleen, where total cell numbers can be up to 10^8, there is sufficient toxin detectable at either time point alone to cause apoptosis. Levels of gliotoxin in the thymus are lower than in the spleen but are high enough to cause apoptosis if gliotoxin does constantly turn over. Thus, gliotoxin does reach lymphoid organs when injected intraperitoneally and probably has a direct action on cells of these organs.

DISCUSSION

Injection of gliotoxin into irradiated mice demonstrated that immunosuppressed mice are more susceptible to the lethal effects of the toxin than control animals. For example, 100 μg of gliotoxin was not lethal to control mice, but 25% mortality

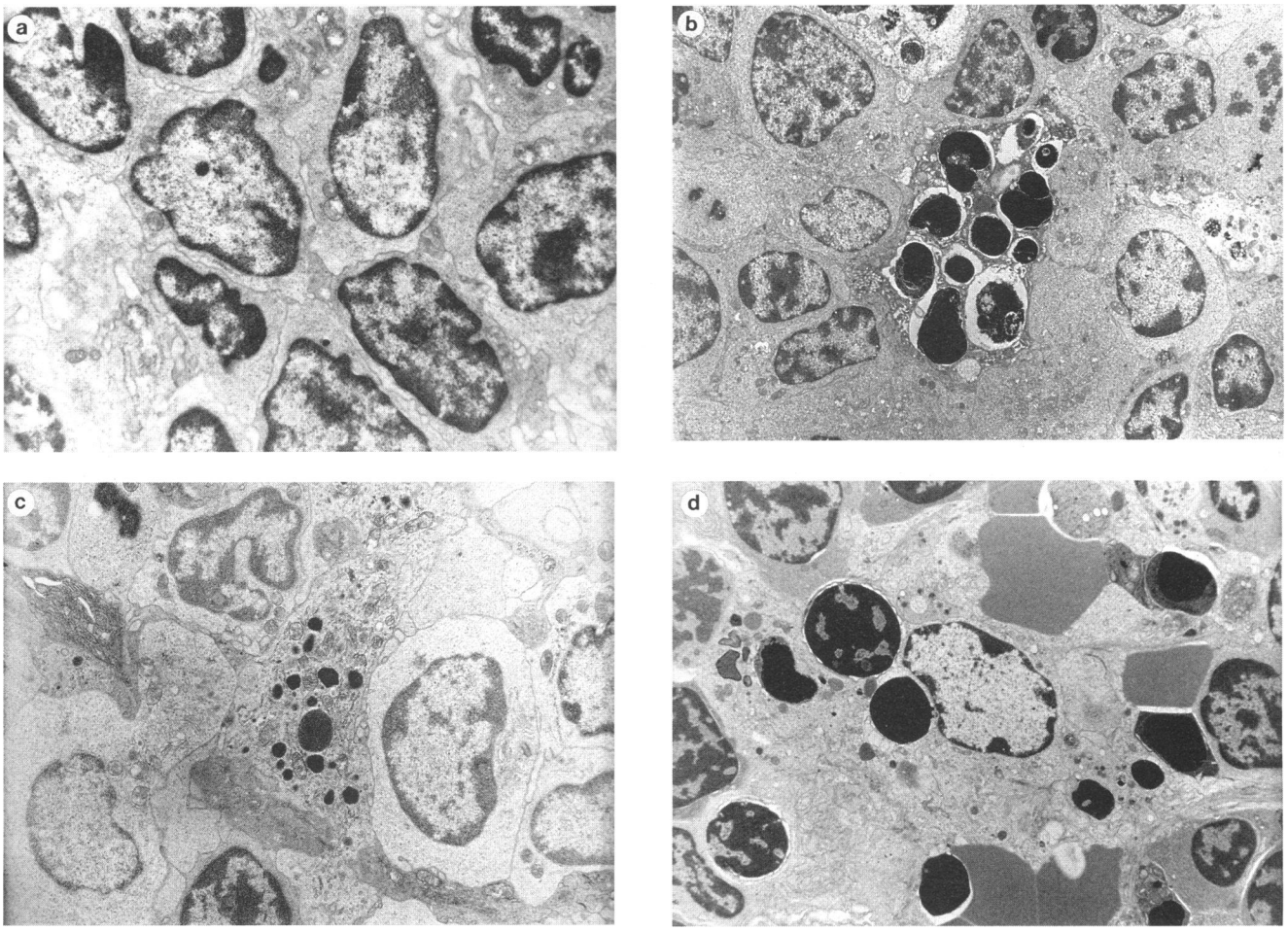

FIG. 4. Electron microscopy of spleen tissue after gliotoxin administration. (a) Control spleen. Gliotoxin was given at 100 μg (b and c) or 200 μg (d and e), and microscopy was done at 6 h (b and d) or 24 h (c and e) after injection. Magnifications, ×2,444 (a, b, c, and e) and ×1,880 (d).

was observed in mice exposed to 6 Gy 5 days earlier. This could have implications for humans receiving radiotherapy who become infected with this fungus.

The proliferation assays and FACS data clearly demonstrate that a single injection of gliotoxin into immunosuppressed mice significantly delayed the recovery of LPS-responsive, mature B lymphocytes in the spleen. Thus, gliotoxin can influence the immune function of mice in vivo. Comparison of Fig. 1b and c shows that the recoveries of LPS and ConA-responsive cells in the spleen occur at roughly the same time in mice injected with gliotoxin (about 30 to 35 days). In irradiated mice which did not receive gliotoxin, normal levels of mature T cells also reappeared in the spleen at around 30 days, whereas LPS-responsive B cells recovered more quickly (at about 15 days). This difference is most likely due to the requirement for T cells to undergo selection in the thymus before appearing in the spleen, but that requirement does not explain why T-cell recovery is not delayed, especially since it has been shown that ConA and LPS responses are equally sensitive to gliotoxin in vitro (10) and since, as reported here, apoptotic foci are evident in the thymus.

Although we have shown here that gliotoxin can reduce the number of B lymphocytes, this capability is unlikely to influence the course of *Aspergillus* infections, as B cells are not thought to play an important role in eliminating this fungus. The most important cell types involved are macrophages, for phagocytosing spores, and neutrophils, for killing *Aspergillus* hyphae (13). Work is currently under way to determine the degree to which gliotoxin can influence the capability of macrophages and neutrophils to fight *Aspergillus* infections.

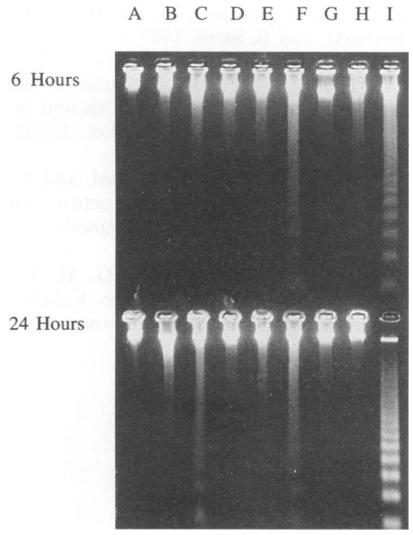

FIG. 5. Agarose gel electrophoresis of DNA isolated from lymphoid organs after gliotoxin administration. Lanes A to C, mesenteric lymph nodes (0, 100, and 200 μg of gliotoxin, respectively); lanes D to F, spleen (0, 100, and 200 μg of gliotoxin, respectively); lanes G to I, thymus (0, 100, and 200 μg of gliotoxin, respectively).

The study of sections of lymphoid organs by light microscopy, electron microscopy, and agarose gel electrophoresis of cellular DNA from cells of those organs demonstrated that injection of gliotoxin causes cells in the thymus, spleen, and mesenteric lymph nodes to undergo apoptosis, and experiments with radiolabelled gliotoxin showed that this is probably due to a direct action of the toxin. The apoptotic cells were often observed in localized foci, predominantly in the thymus and germinal centers of the spleen. It is possible that recirculating lymphocytes carried gliotoxin from the peritoneum to the lymphoid organs, where they released the metabolite, which then affected neighboring cells. Release of biologically active gliotoxin from cells of hemopoietic origin has been demonstrated in vitro (22). However, intraperitoneal injection of splenocytes or peritoneal cells, treated ex vivo with gliotoxin, did not result in similar apoptotic foci (data not shown). Another possibility is that gliotoxin exerts a selective action on cells depending upon their current stage in the cell cycle. The location of the apoptotic foci correlates with sites of accelerated mitosis in the thymus and germinal centers of the spleen and lymph nodes.

A surprising observation was that of the appearance of apoptotic cells and bodies inside macrophages, as seen by electron microscopy, in the organs of mice injected with gliotoxin (Fig. 4c). It has been shown previously that gliotoxin inhibits phagocytosis by macrophages at rather low concentrations (9). This suggests either that no active gliotoxin was present at the time of phagocytosis or that macrophages have a lower sensitivity to gliotoxin in vivo than lymphocytes.

In conclusion, we have demonstrated that gliotoxin, a metabolite produced in vivo during pathogenic fungal infection, has greater toxicity after sublethal irradiation, affects the recovery of B lymphocytes in immunocompromised mice, and causes apoptosis in primary and secondary lymphoid organs.

TABLE 2. Amount of injected gliotoxin reaching mouse lymphoid organs[a]

Organ and amt (μg) of injected gliotoxin	Amt (μg) of gliotoxin (concn [μM]) after[b]:	
	6 h	24 h
Spleen (100)	125 (5)	38 (1.5)
Spleen (200)	266 (11)	230 (9)
Thymus (100)	12 (0.5)	48 (2)
Thymus (200)	42 (2)	50 (2)

[a] Radioactivity in homogenized organs was measured in a scintillation counter and expressed as a percentage of the total radioactivity injected.

[b] Gliotoxin in each organ was calculated as percent radioactivity times the total amount of gliotoxin injected (assuming ^{35}S was still present in association with gliotoxin). Values in parentheses are the effective concentrations of gliotoxin in the organ, based on a volume of 80 μl (estimated by weight and water displacement).

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