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Effects of Immunoglobulin G and Low-Dose Amphotericin B on *Candida albicans* Infections in Burned Mice

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Candidiasis causes serious problems for compromised hosts. Effective treatments for *Candida albicans* infections are few. To see if immunoglobulin (Ig) therapy could be beneficial, burn-immunocompromised mice were treated intravenously with 2.5 mg of five different IgG preparations 48 h postburn and post-*C. albicans* challenge. Despite up to fourfold differences in titer (1:1,600 to 1:6,400) to *C. albicans*, all preparations improved 10-day survival about 30% ($P < 0.0001$). Treatment with a low dose of amphotericin B (AmB; 0.09 mg/kg of body weight) intravenously 24 and 48 h after burn and challenge improved survival 9 to 45% ($P < 0.0001$). Treatment with a low dose of AmB plus IgG showed the same results as treatment with AmB alone and better results than treatment with IgG alone. Quantitative renal cultures from burned mice treated with AmB plus one IgG preparation, Sandoglobulin, showed that *C. albicans* counts decreased in sham-treated mice from 7.21 ± 0.15 $\log_{10}$ CFU/g (mean ± standard error of the mean) to 5.31 ± 0.34, which was significantly less than counts with AmB (6.11 ± 0.35) or Sandoglobulin (6.39 ± 0.18) treatment alone. We conclude that (i) by using decreases in mortality and in renal fungal load as end points, treatment with IgG preparations alone or with a low dose of AmB alone protected burn-immunocompromised mice from candidiasis; (ii) though AmB plus one IgG preparation significantly decreased renal fungal load, the combination did not significantly decrease mortality beyond that found with AmB alone; and (iii) survival data did not correlate with IgG titers to *C. albicans*.

Invasive candidiasis is a life-threatening problem for immunosuppressed patients, such as those undergoing transplantation, AIDS patients, low-birth-weight babies, cancer patients, and burn victims (6). There are few effective therapies against this infection (24). Of the treatments available, the polyene antifungal agents, amphotericin B (AmB), is the most widely used (20). However, AmB therapy has significant, dose-dependent, negative side effects (17).

While it is well recognized that cellular immunity is of major importance in host protection against candidiasis (17, 18), there are suggestions that humoral immunity may also play a role (1, 10, 12, 18). For example, Cline and Lehrer (1) have shown that immunoglobulin G (IgG) can serve as an opsonin for the phagocytosis of *Candida albicans*, and passive transfer of serum from immune animals has been reported to protect mice against *C. albicans* (12, 18). Conversely, Maite et al. (10) have demonstrated that newborn mice made deficient in B lymphocytes by treatment with anti-mouse μ serum have enhanced susceptibility to *C. albicans* challenge. In both human burn patients (7, 13, 22) and animals (14) immunocompromised by a burn and infected with *C. albicans*, immunoglobulin levels are low. For the past decade, there have been a number of IgG formulations, prepared by a variety of methods, available for intravenous use. Some of these preparations have been shown to have titers against *Candida* species (19). Since human IgG preparations have been shown to have both specific and nonspecific effects on immunity in murine models of bacterial infection (4), we used a burn-compromised mouse model and five different IgG preparations to investigate the following questions about *C. albicans* infections. Will IgG therapy improve the survival of burn-compromised hosts which are infected with *C. albicans*? Will IgG therapy improve the efficacy of a low, subclinical dose of AmB in protecting burned mice against *Candida* infection? Is the IgG titer to *C. albicans* proportional to the protection seen in the burned *C. albicans*-infected mice treated with IgG?

MATERIALS AND METHODS

Mice and burn procedure. Female, 22- to 24-g, Crl-CF1 BR non-Swiss mice from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) were housed five mice per cage and given standard mouse chow and water ad libitum. The burn procedure, which produces a partial-thickness nonlethal burn of 12 to 15% of the total body surface area, was performed as described previously (16). Briefly, the backs of the mice were shaved, after which the mice were anesthetized by inhalation of Metofane (Pitman-Moore, Inc., Washington Crossing, N.J.). A heat-resistant plastic board with a 1 by 1.5 in. (2.54 by 3.81 cm) window was pressed against the shaved back, and 0.5 ml of ethanol was spread in the opening of the window, ignited, and allowed to burn for 10 s. Immediately postburn, 0.5 ml of normal saline was injected intraperitoneally as fluid replacement therapy. Sham treatment consisted of all the above manipulations, with the exception of the burn. Four or five mice were included in each group, and the results from repeated trials were pooled.

***C. albicans* inoculum preparation and challenge.** *C. albicans* MY 1044 (serotype B), obtained from Ann Edison (Merck & Co., Inc., Rahway, N.J.), was used in all experiments. MIC testing (21) indicated that this *C. albicans* strain was sensitive to AmB (MIC = 0.2 μg/ml, with a MIC of <1.0 μg/ml indicating sensitivity).

The *C. albicans* was grown in brain heart infusion broth in a shaking water bath at 37°C for 17 h, after which cell density was adjusted with phosphate-buffered saline (PBS) to 10^7

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cells per ml with a Klett-Summerson colorimeter. A 10-fold dilution with PBS gave the desired inoculum dose of 10^6 cells per ml. Actual viable cell counts were determined by performing serial dilution plate counts in duplicate. The average challenge inoculum (mean ± standard error) was (4.1 ± 0.4) × 10^6 cells per ml. At 1 h postburn, 0.1 ml of the challenge inoculum was given via a tail vein.

Treatments. AmB (Fungizone [intravenous]; Squibb, Princeton, N.J.) was given intravenously in two doses of either 2.0 μg per mouse (0.09 mg/kg of body weight, low dose) or 10.0 μg per mouse (0.45 mg/kg of body weight, clinical dose) at 24 and 48 h postburn. Doses were predicated upon the recommended patient dose in milligrams per kilogram of body weight adjusted to the weight of an approximately 23-g mouse.

Five different human IgG preparations were used: Gamimune (Cutter Biologicals, Miles Laboratory, Inc., Berkeley, Calif.), Gammagard (Hyland-Baxter Laboratories, Inc., Glendale, Calif.), Sandoglobulin (Sandoz Pharmaceuticals Corp., East Hanover, N.J.), and two IgG preparations from Armour Pharmaceuticals Co. (Ft. Washington, Pa.). The Armour products, a normal IgG and a *Pseudomonas* hyperimmune IgG, have been extensively defined previously (15). Briefly, the Armour normal IgG is human IgG pooled from a large group of normal, i.e., nonimmunized, donors, as are Gamimune, Gammagard, and Sandoglobulin. In contrast, the *Pseudomonas* hyperimmune IgG is an IgG preparation from high-titered sera of human responders who were specifically immunized with a 16-valent *Pseudomonas*-extract vaccine. All lyophilized preparations were reconstituted according to the suppliers' instructions (sterile saline for Sandoglobulin and sterile water for the others); for any further dilutions, sterile saline was utilized. All preparations were reconstituted at least 2 h prior to use to ensure complete hydration of the protein. Each preparation was injected intravenously in doses of 2.5 mg of IgG in a 0.1-ml volume at 48 h postburn. Two different sham treatments were tested: saline injection (0.1 ml) and injection of 2.5 mg of albumin (normal serum albumin [human], 25% USP; Cutter Biologicals, Miles Laboratory, Inc.) in a 0.1-ml volume (as a protein control).

Determination of Ig preparation titers. All Ig titers were determined by Michael S. Collins (Cutter Biologicals, Miles Laboratory, Inc.). *C. albicans* B311, serotype A, and 526B, serotype B, were cultured overnight on glucose (1%)–yeast extract (0.5%)–agar (1.5%). Cells were washed two times with PBS and adjusted to an A_{660} of 0.20. Yeast cells (100 μl) were incubated at 37°C for 30 min with an Ig sample that had undergone multiple twofold dilutions (1:100 to 1:12,800) with saline. Goat anti-human IgG-IgA-IgM serum (fluorescein isothiocyanate conjugated) (Hyclone Laboratories, Logan, Utah) was diluted to 1:30. Fluorescein isothiocyanate (50 μl) was added to washed yeast cells. After 30 min of incubation at 37°C, cells were washed once with PBS and fluorescence was assessed.

Renal *C. albicans* quantitations. Both kidneys were removed aseptically, weighed, placed in 10 ml of sterile normal saline in a Dounce homogenizer (Kontes, Vineland, N.J.), and ground to a homogeneous solution with a drill press. All samples were kept in an ice bath until they were serially diluted and plated in duplicate onto Sabouraud dextrose agar (Becton Dickinson and Co., Cockeysville, Md.). After 48 h at 37°C, colonies were counted, and the results were expressed as the mean ± standard error for all mice receiving any given treatment. Four or five mice were included in each group per each experiment, and each experiment was run three times for a total of 12 or 15 mice per group.

TABLE 1. Effect on survival of treating burned mice challenged with *C. albicans* with various IgG preparations and/or a low dose of AmB

Treatment	Survival at day 10 [no. of mice alive/no. of mice tested (%)]
None (sham)	5/53 (9)
Gamimune	9/23 (39)
Gammagard	8/20 (40)
Pseudomonas hyperimmune IgG	8/19 (42)
Normal IgG	10/23 (43)
Sandoglobulin	8/20 (40)
Total	43/105 (41)[a]
AmB	21/47 (45)[a]
AmB + Gamimune	12/27 (44)
AmB + Gammagard	12/20 (60)
AmB + *Pseudomonas* hyperimmune IgG	15/26 (58)
AmB + Normal IgG	13/26 (50)
AmB + Sandoglobulin	16/20 (80)
Total	68/119 (57)[a]

[a] Significantly greater than that of sham treatment results by unordered exact test, $P < 0.0001$.

Statistics. Statistical differences between mortality figures were determined by using an unordered exact contingency table test (STATXACT, Cambridge, Mass.). Since multiple groups were compared, P values were not considered significant unless they were less than 0.05, the normal significance level divided by the number of possible comparisons which could be made among the groups analyzed. For example, in analyzing data from three possible pairings, we required P to be less than 0.05 divided by 3, i.e., <0.0167, before any pairwise comparisons were considered significantly different. Statistical differences among the means of quantitative culture data were determined by ANOVA followed by Duncan's multiple comparison test, in which case differences were considered significant when P was <0.05.

RESULTS

Titers. Each IgG preparation had measurable titers against *C. albicans*. Since the titers against the A serotype strain were exactly the same as those against the B serotype, only one set of titer data is given for the IgG preparations: 1:1,600 for Gamimune, 1:3,200 for Gammagard, 1:6,400 for Ps hyperimmune IgG, 1:3,200 for normal IgG, and 1:1,600 for Sandoglobulin. The highest titer against *C. albicans* occurred with the *Pseudomonas* hyperimmune IgG preparation (1:6,400), and lower titers were measured for the other four IgG preparations.

Protection studies. Initial experiments indicated that groups of mice given no treatment, saline sham treatment, or the albumin protein control sham treatment all had similar survival results (0 to 20% at day 10). Therefore, subsequent experiments utilized only a saline sham-treated group (Table 1). Burned mice challenged with *C. albicans* and treated with any of the five different IgG preparations showed a consistent survival of approximately 40% (Table 1). Unordered exact test analysis of these results for the five different IgG treatment groups indicated no differences in survival for the different IgG preparations tested ($P = 0.99$). Hence, all IgG

treatment data were pooled, with the result that IgG therapy improved survival of sham-treated animals from 9 to 41% (Table 1; $P < 0.0001$).

As recommended by the manufacturer, treatment of humans with AmB generally starts at 0.25 mg/kg of body weight and is then increased to daily doses as high as 1.0 mg/kg of body weight. We found 100% protection at 10 days postburn and postchallenge with two doses, one on day 1 and one on day 2, of 0.45 mg of AmB per kg of body weight (10 μg per mouse). To determine a low (subclinical) dose of AmB, dose-response experiments were done. Decreasing the AmB dose to 0.09 mg/kg of body weight (2.0 μg per mouse) gave only partial protection (45% survival for low-dose-AmB-treated mice versus 9% survival for sham-treated animals [Table 1], $P = 0.0001$). Treating burned *C. albicans*-challenged mice with one of the IgG preparations in addition to the low dose of AmB gave survival results of 44% (Gamimune) to 80% (Sandoglobulin). Unordered exact test analysis of mortality results from these five different IgG plus AmB groups showed no significant differences in effect among the five groups ($P = 0.15$). Hence, the mortality results for all AmB-plus-IgG-treated groups were pooled, with a survival of 57%, which was statistically different from the 41% survival ($P < 0.0001$) for animals treated with IgG alone but not significantly different from the 45% survival ($P = 0.10$) for mice treated with a low dose of AmB alone.

Quantitative cultures. To determine whether treatment with IgG (Sandoglobulin) and/or the low dose of AmB affected renal *C. albicans* census, mice were sacrificed 4 days postburn and postchallenge (a time just prior to the time when the untreated, sham mice began to die), and *C. albicans* counts in renal cultures were quantitatively determined (mean $\log_{10}$ CFU/g ± standard error of the mean). Both Sandoglobulin (6.39 ± 0.18, $n = 15$) and AmB (6.11 ± 0.35, $n = 13$) treatments alone significantly decreased renal *Candida* census. The treatments together (5.31 ± 0.34, $n = 14$) decreased the quantitative culture census even further. The result for the sham-treated mice was 7.21 ± 0.15 ($n = 15$).

DISCUSSION

Since *C. albicans* is a relatively ubiquitous organism, and since all of the IgG preparations in this study were separated from pooled plasma from a large number of volunteers, it is not surprising that all five preparations tested had titers against *C. albicans*. These titers are in agreement with the results from an earlier study by Rousell et al. (19), which showed that titers to *Candida* species existed in a previous Gamimune preparation which was prepared for intravenous use by reduction and alkylation rather than by pH 4.25 adjustment, as was the Gamimune preparation used in this study (11). In the study by Rousell et al. (19) of Gamimune, only titers against *Candida* species were measured, while in this study antibody titers directed specifically against *C. albicans*, both serotypes A and B, were determined for five IgG preparations. While approximately two times more *C. albicans* serotype A strains than serotype B strains are isolated clinically (17), titers against both serotypes in all IgG preparations tested were equal.

Among the five IgG formulations tested, the highest titer against *C. albicans* occurred in the *Pseudomonas* hyperimmune IgG preparation. It is of interest that this Armour preparation, which was extracted from plasma from volunteers who showed strong antibody production when immunized with *Pseudomonas aeruginosa* antigens (15), also showed the highest *C. albicans* antibody levels in our test preparations, suggesting perhaps that the volunteers used for the *Pseudomonas* hyperimmune IgG plasma pool were, in general, strong antibody producers or that *Pseudomonas* vaccination produced a nonspecific increase in antibody production (25).

Regardless of antibody titers, each of the five IgG preparations, when given on an equal-weight basis (2.5 mg per mouse), provided equal protection to burned mice challenged with *C. albicans*. Hence, in vitro titers did not correspond with in vivo protection. This is not a new finding, at least with regard to IgG titers and protection against bacterial infections (2, 16, 19). In vitro titers and protection in animals may not correlate because the titers simply indicate the presence of a particular molecule but do not give any indication of whether the molecule is functional relative to protection. Collins and Dorsey (2) suggested that the most useful test for evaluating the functional activity of an IgG preparation in protecting against bacterial infection is a well-controlled model of experimental animal infection. The same appears to be true for evaluating the protective capacity of IgG preparations against fatal candidiasis.

IgG production differences, such as donor selection, plasma fractionation methodology, and stabilizers used, can affect the biological activity of the final IgG product (8, 9, 26). Despite the methodological differences in formulating one IgG preparation versus another, all IgG products tested in this study showed very similar moderate improvement in survival when used to treat burned mice challenged with *C. albicans* (Table 1).

How IgG treatment protected the burned host remains to be determined. The increase in survival and the decrease in *C. albicans* census in the IgG-treated mice could be related to specific or nonspecific effects of IgG on the host's immune system. That the IgG itself caused these effects seems indicated, since injections of another fluid (saline) or a protein (albumin) were not efficacious. However, Drouhet et al. (5) have shown that causing an acute nonspecific inflammatory reaction with calcium pyrophosphate or dextran injection 1 to 7 days before *Candida* challenge protected mice for 2 to 5 days from *Candida*-caused deaths. Perhaps the IgG also causes a nonspecific protection. The findings that all IgG preparations, regardless of their titers against *C. albicans*, protected the burned mice equally would support such a nonspecific effect of IgG on host immunity. Studies to elucidate whether the fungal elements in the host are in the blastoconidial or pseudohyphal forms may help determine whether the IgG is acting as an opsonizing agent or in some other capacity, perhaps by countering a *Candida* extracellular protease which could be acting as a virulence factor. Clearly, further studies to elucidate the mechanism of the protective effects of IgG against candidiasis are needed.

As with any animal study, caution needs be taken in extrapolating these results either to other species, such as man, or to other immunocompromising situations, such as cancer. In 1984, Cross et al. (3) reported overwhelming *C. albicans* sepsis following administration of IgG to a patient with myelocytic leukemia and warned that safe use of IgG for one group of patients, such as hypogammaglobulinemics, is no guarantee of safety for other groups of patients. Subsequently, however, IgG preparations have been given to a wide variety of patient populations. In an editorial in the New England Journal of Medicine in 1991, Stiehm (23) reviewed IgG use and stated that "these preparations are safe. Immediate adverse reactions are unusual, usually predictable and easily treated." The author did point out that

while there are a growing number of situations in which giving IgG treatment is indicated, there are also situations in which IgG is either not efficacious or not cost effective. Whether any patient population with candidiasis can be treated effectively, safely, and in a cost-responsible manner with IgG preparations remains to be determined.

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