INFECTION AND IMMUNITY, July 1985, p. 172–181
0019-9567/85/070172-10$02.00/0

Vol. 49, No. 1

Immunoregulation in Experimental Murine Candidiasis: Specific Suppression Induced by *Candida albicans* Cell Wall Glycoprotein

EMILY W. CARROW†* AND JUDITH E. DOMER

Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, Louisiana 70112

Received 10 December 1984/Accepted 8 April 1985

Immune regulation in candidiasis is inferred from studies of both human and animal infection, with a suppressive role suggested for cell wall polysaccharide. To study the immunosuppressive potential of *Candida albicans* in a murine model, whole blastoconidia or purified cell wall components of *C. albicans* were tested for their effects on the development of acquired immune responses by superimposing a pretreatment regimen upon an established immunization protocol. CBA/J or BALB/cByJ mice were pretreated twice intravenously with 100 μg of mannan (MAN), 100 or 200 μg of glycoprotein (GP), or 5×10^7 heat-killed *C. albicans* blastoconidia, followed 1 week later by an immunization protocol of two cutaneous inoculations of viable *C. albicans* blastoconidia given 2 weeks apart. Delayed hypersensitivity (DTH) to GP or to a membrane-derived antigen, B-HEX, was tested 7 days after the second inoculation, and lymphocyte stimulation was tested with mitogens and *Candida* antigens after 12 days. To assess protection, mice were challenged intravenously with viable *C. albicans* blastoconidia 14 days after the second cutaneous inoculation and sacrificed 28 days later for quantitative culture of kidneys and brains. Sera were obtained for enzyme-linked immunosorbent assays at selected intervals. Pretreatment with GP resulted in specific in vivo suppression of DTH to GP but not to B-HEX antigen and specific in vitro suppression of lymphocyte stimulation to GP but not to other *Candida* antigens or mitogens. MAN and heat-killed *C. albicans* blastoconidia had no such effects. GP pretreatment also diminished the protective effect of immunization against challenge, demonstrable in the brain, while not altering significantly the production of antibody in response to infection. Contrary to clinical evidence, MAN was not immunosuppressive in this model, and in fact, the immunosuppressive potential of GP, which is composed largely of MAN, was found to be dependent upon the presence of its heat-labile protein moiety.

Immunosuppression appears to be relatively common during the clinical course of severe systemic mycotic disease (1, 7, 11, 21, 40). In candidiasis, the clinical evidence indicates that a factor of some sort, perhaps a fungal constituent or product, is responsible for the immunosuppression observed. For example, a number of investigators (2, 13, 15, 21) have noted an inhibitory factor in the serum of patients with chronic mucocutaneous candidiasis which appears to suppress both in vivo and in vitro responses to *Candida* antigens. It has been suggested (13, 14, 28, 44) that the inhibitory factor in serum is mannan (MAN), a major surface antigen. Indeed, MAN antigenemia can be detected during disseminated candidiasis and is often associated with a poor prognosis. (See reference 10 for a review.)

Experimental models of candidiasis have provided some evidence of a suppressive potential for *Candida* blastoconidia (35–39, 43), but with one exception (38), no attempts have been made to identify which components of the organism were responsible for the effect; moreover, these studies were designed to examine suppression of innate responses only, with animals not previously sensitized to *C. albicans*.

The murine model of candidiasis developed in this laboratory is an attempt to approximate more closely the immune status of normal healthy adults with regard to *C. albicans*, including development of *Candida*-specific delayed hypersensitivity (DTH), production of specific antibody, protection against reinfection, and lymphocyte responsiveness to *Candida* antigens in vitro (12, 16, 26). The model has proven useful in our investigations of acquired immunity to *C. albicans* (6, 17, 19, 25), and it has been manipulated for the study reported here to investigate the potential for circulating antigens of *C. albicans* to alter the development of normal immune responses to local infection. Specifically, we chose to study the effects of intravenous inoculation of whole killed blastoconidia or purified cell wall components (MAN or glycoprotein [GP]) on the acquired immune response to *C. albicans* by superimposing a pretreatment regimen upon an established immunization protocol. Cell wall MAN was chosen for study on the basis of clinical evidence of MAN-mediated immunosuppression mentioned earlier. Although there is no evidence for a similar capacity of GP, it has been implicated in the toxicity of *C. albicans* (8) and in pharmacological events leading to histamine release (30, 41).

MATERIALS AND METHODS

Mice. Male CBA/J or BALB/cByJ mice (6 to 8 weeks old) were obtained from Jackson Laboratory, Bar Harbor, Maine. CD-1 mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. All mice were maintained under conventional conditions and fed mouse chow and water ad libitum.

Culture methods. *C. albicans* B311, originally obtained from H. Hasenclever, was used throughout these studies. It was maintained at 4°C by monthly transfer on Sabouraud dextrose agar. At regular intervals, lyophilized preparations were rehydrated to ensure the maintenance of virulence. Viable blastoconidia for inoculation into mice were incubated in tryptic soy dialysate broth (34) for 18 h at 37°C on a gyratory shaker operating at approximately 165 rpm before

* Corresponding author.
† Present address: The North Carolina Memorial Hospital, University of North Carolina, Chapel Hill, NC 27514.

harvesting by centrifugation. After three washes with sterile nonpyrogenic saline (NPS; Cutter Laboratories, Inc., Berkeley, Calif.), the blastoconidia were counted in a hemacytometer and suspended in NPS at the desired concentration. Each suspension was further diluted and spread onto Sabouraud agar plates to determine the viable count. Heat-killed *C. albicans* blastoconidia (HKC) were prepared by heating an 18-h broth culture for 2 h at 60°C before harvesting as described above. Loss of viability was confirmed by plating onto Sabouraud agar. Viable blastoconidia from which antigens were prepared were harvested from soy dialysate broth as above, but after harvest they were washed four times with 0.15 M phosphate-buffered saline (pH 7.2) containing 0.001 M phenylmethylsulfonyl fluoride and stored at −20°C in the same buffer until fractionated.

Fractionation procedures and source of test antigens. MAN was extracted from whole blastoconidia in citrate buffer by the method of Peat et al. (32) as modified by Kocourek and Ballou (22). Other subcellular components were prepared from the blastoconidia suspended in 0.15 M phosphate-buffered saline containing 0.001 M phenylmethylsulfonyl fluoride by disruption with ballistic action in a Braun homogenizer followed by differential centrifugation as described previously (12). The three fractions used in this study are designated B-HEX, SCS, and GP. The SCS, soluble cytoplasmic substances, was the dialyzed and lyophilized supernatant remaining after a final centrifugation at 144,000 × *g* for 2 h. B-HEX was prepared from a membrane-mitochondria fraction which had been extracted with butanol and hot phosphate-buffered saline (12, 25) before the precipitation of protein with 100% saturated ammonium sulfate. The precipitate was redissolved in and dialyzed against NPS and stored at −70°C. Protein content was determined by the method of Lowry et al. (24) with bovine serum albumin as a standard. GP was a water-soluble cell wall fraction which had been extracted with ethylenediamine before precipitation with methanol (23). GP resulting from this procedure was used as such, and in addition, portions were either heated for 1 h at 65°C or treated with papain (Sigma Chemical Co., St. Louis, Mo.) as previously described (12) to assess the role of protein in responses elicited by GP. Briefly, 30 mg of GP was incubated for 4 h at 37°C in 6 ml of an acetate–EDTA–2-mercaptoethanol buffer to which freshly prepared L-cysteine was added. It was then heated for 1 h at 65°C to inactivate the enzyme, dialyzed, and lyophilized.

Animal inoculations. In experiments involving pretreatment with antigen, mice were injected intravenously via the lateral tail vein with one of the following preparations contained in 0.5 ml of NPS: MAN, 100 μg; HKC, 5×10^7 blastoconidia; GP, 100 μg; GP, 200 μg; heat-treated GP, 100 μg; papain-treated GP, 200 μg; heat-treated papain, 100 μg. Immunization of mice was accomplished by the intracutaneous inoculation of 10^6 viable *C. albicans* blastoconidia suspended in 0.05 ml of NPS as described previously (16). When testing for the development of a protective response, control and immunized animals were challenged 2 weeks after the second cutaneous inoculation by administering intravenously via the lateral tail vein 10^4 viable blastoconidia suspended in 0.5 ml of NPS. Animals were monitored for mortality for 28 days, bled, and then sacrificed for quantitative culture of brains and kidneys.

Footpad testing. A 0.02-ml portion of test preparation, either B-HEX adjusted to 20 μg of protein per test dose or GP in NPS at 30 μg per test dose, was injected into the footpad with a micrometer syringe. Footpads were measured with Schnelltaster calipers (H. Kroplein GmbH, Schluchtern, Federal Republic of Germany) before the injection of antigen. At 15 min and 4, 7, 24, and 48 h after injection, footpads were remeasured, and the mean net increase in thickness was determined for each group. In each experiment the animals were coded, randomized, and injected by one individual and then measured by a second individual to whom the code was unknown. Unimmunized animals were used as controls.

Lymphocyte stimulation. Proliferative responses of cells from inguinal lymph nodes draining the site of cutaneous inoculation were assayed by a micromethod described previously (19). The mitogens used were phytohemagglutinin P (PHA) (Difco Laboratories, Detroit, Mich.) at 1:50, 1:100, or 1:200 dilutions from stock; pokeweed mitogen (PWM) (GIBCO Laboratories, Grand Island, N.Y.) at 1:25, 1:50, or 1:100 dilutions from stock; and concanavalin A (ConA) (Calbiochem-Behring, La Jolla, Calif.) at 0.5, 1.0, or 1.25 μg per well. Antigens used were B-HEX at 10, 20, or 40 μg of protein per well; SCS at 50, 100, or 200 μg of protein per well; and GP at 50, 100, or 200 μg per well. Mitogen-stimulated cultures were harvested after 3 days of incubation, and those stimulated with antigens were harvested after 4 days, each having been pulsed with 0.5 μCi of [*methyl*-^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) 18 h before harvest. Results are expressed as the mean counts per minute for triplicate cultures of treated cells minus those of untreated cells.

Antibody detection. Anti-*Candida* antibody in the sera of mice was determined by a solid phase, double antibody enzyme-linked immunosorbent assay described previously (19). SCS or GP was used as the *Candida*-specific antigen, bovine serum albumin was used as a blocking agent, and peroxidase-labeled goat anti-mouse immunoglobulin G plus immunoglobulin M (heavy- and light-chain specific) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was used as the enzyme-antibody conjugate. Values for representative positive control results were plotted as optical density at 405 nm versus log titer to determine the 50% endpoint value, and the corresponding optical density value was used to determine titers.

Calculations and statistical analyses. The total number of CFU per organ was determined by standard dilution calculations and was expressed as logarithmic numbers. Mean values for kidneys or brains were calculated within each group. Because deaths before the specific time of sacrifice would bias such results, a normalization factor representing the maximum values seen for each organ was included in the calculations for each animal that died. Where applicable, data were analyzed by Student's *t* test. Significance was determined at the $P = 0.05$ level.

Experimental design. The manner in which the mice were immunized and the times selected at which the various immune parameters were measured were based on previous studies with the CBA/J mouse (12, 16, 26). Mice were immunized by the cutaneous inoculation of 10^6 viable *C. albicans* blastoconidia administered 2 weeks apart. DTH responses were detected 7 days and lymphocyte stimulation responses 12 days after the second cutaneous inoculation. Intravenous challenge to assess protection was administered 14 days after the second cutaneous inoculation, and protection was assessed by culture of kidneys and brains 4 weeks thereafter. Animals receiving pretreatments were injected intravenously with one of the antigen preparations either 1 week or 1 and 2 weeks before immunization unless otherwise indicated. Blood drawn before immunization, after final

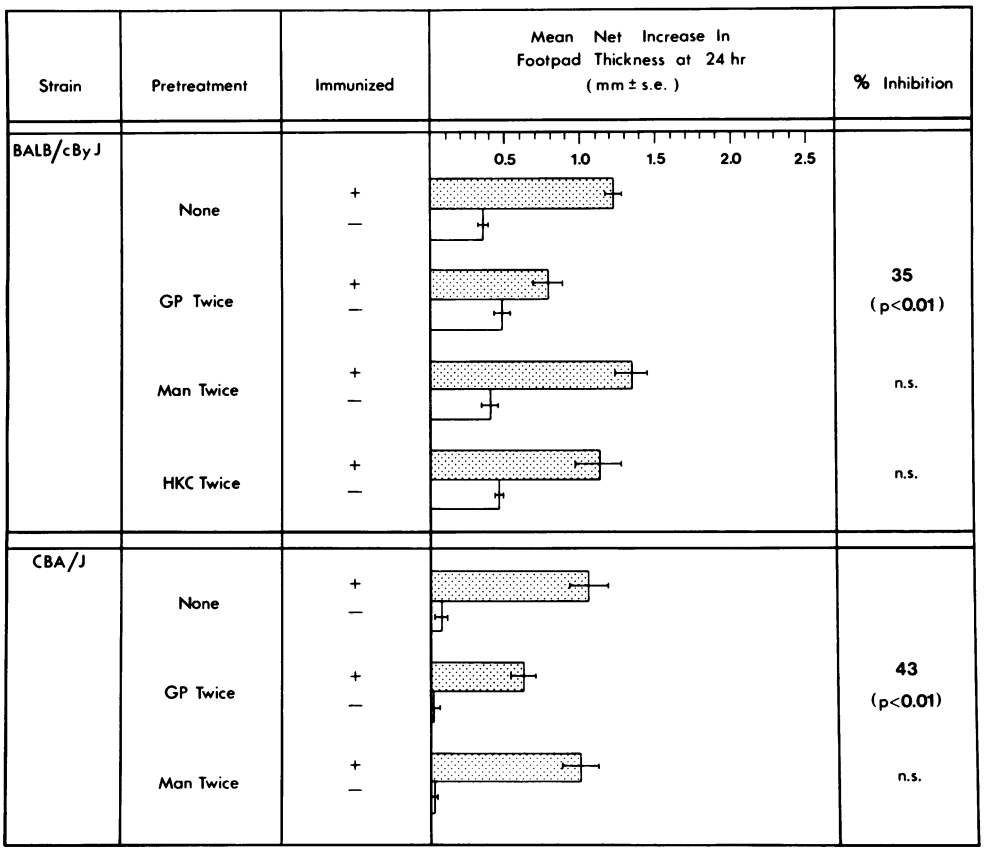

FIG. 1. Footpad responses to 30 μg of cell wall GP in immunized (stippled bars) and unimmunized (open bars) mice pretreated by intravenous inoculation of 100 μg of GP or MAN or 5×10^7 HKC either twice at weekly intervals before immunization with viable *C. albicans* (BALB/cByJ) or once before and once after the initiation of the immunization protocol (CBA/J). Percent inhibition = (increase in thickness for immunized unpretreated mice − increase for immunized pretreated mice)/(increase for immunized unpretreated mice) × 100. n.s., No significant difference. $n = 7$.

footpad measurements, and at the time of sacrifice was assayed for antibody. At least three separate experiments were performed with similar results. Data from representative experiments are presented.

RESULTS

Effect of pretreatment on the development of cellular immunity demonstrable in vivo. Mice that were pretreated once or twice with GP, MAN, or HKC were footpad tested with GP 7 days after immunization by two cutaneous inoculations of *C. albicans*. The results from two experiments in which BALB/cByJ and CBA/J mice were tested are summarized in Fig. 1. Although footpads were measured at five intervals over a 48-h period, only the 24-h response is shown, since that was the time of the maximum DTH reaction. In all footpad assays, the temporal development of responses was similar to that shown in Fig. 2. The data in Fig. 1 illustrate that intravenous pretreatment with GP had a suppressive effect on the DTH response to GP antigen, while pretreatment with MAN or HKC had no such effect. BALB/c mice pretreated twice with GP and CBA/J mice treated once before and once after immunization both had significantly lower responses than unpretreated immunized controls. CBA/J mice given 200-μg doses of GP before and after immunization were virtually unresponsive to GP antigen (72% inhibition, data not shown). In addition, as this and subsequent experiments showed, pretreatment with any of the antigens in the absence of immunization did not induce *Candida*-specific DTH.

The suppressive potential of GP pretreatment was investigated further to determine a dose-response relationship. CD-1 mice were given 5, 25, or 125 μg of GP intravenously 1 week and 2 weeks before immunization and then footpad tested with GP 7 days after the second cutaneous inoculation. Only those mice that received 125 μg of GP had DTH responses significantly lower than those of unpretreated immunized controls (28% inhibition), although the group receiving 25 μg of GP was also somewhat suppressed (21% inhibition). Pretreatment with 5 μg of GP had no effect (data not shown). A standard dose of 100 μg of GP was chosen for all subsequent pretreatments because injection of greater amounts had a noticeably toxic effect, often leading to shock and premature death. This dose adequately and reproducibly inhibited the DTH response to GP in BALB/cByJ mice, so the BALB/cByJ strain was selected as the most appropriate one for investigating immunoregulatory effects.

To examine the specificity of the effect in terms of DTH responses to different *Candida* antigens, mice were pretreated with GP 1 week or 1 and 2 weeks before immunization and then footpad tested with either GP or B-HEX 7 days after the second cutaneous inoculation. Both GP and B-HEX have proven to be useful in demonstrating DTH in the murine model of candidiasis developed in this laboratory (12). The data are summarized in Fig. 2. As expected, mice pretreated twice with GP exhibited marked suppression of

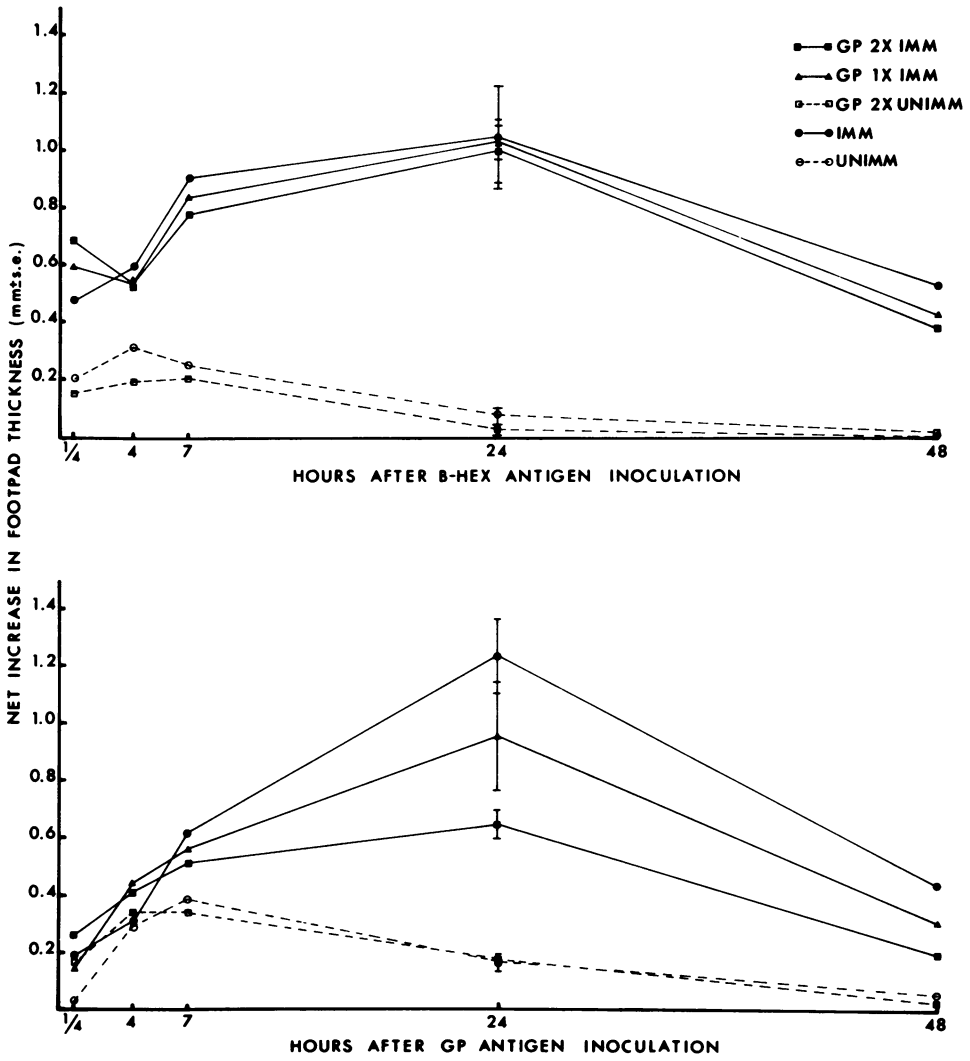

FIG. 2. Footpad responses to B-HEX (20 μg of protein per test dose) or GP (30 μg per test dose) in immunized (solid lines) and unimmunized (dashed lines) BALB/c mice. Pretreated mice received 100 μg of GP intravenously 1 week (GP 1X) or 1 and 2 weeks (GP 2X) before immunization. $n = 7$.

their response to GP (48% inhibition) compared with immunized controls. Mice given only one pretreatment also appeared to be somewhat suppressed (23% inhibition), but the suppression was not significant. On the contrary, all pretreated immunized mice had completely normal responses to B-HEX, indicating that the suppressive effect of GP pretreatment was antigen specific.

Previous examination of GP indicated that its antigenicity was labile to protease treatment (12). To determine whether the protein moiety of the GP was essential to the suppressive potential of GP, mice were pretreated twice with papain-treated GP or, as controls, intact GP, heat-treated GP, or heat-treated papain, then immunized, and footpad tested. The 24-h responses (Fig. 3) indicate that heat treatment considerably reduced the suppressive capacity of GP, while protease treatment abrogated it entirely. Papain alone did not elicit a response. These results strongly implicate the protein portion in the suppressive activity of GP despite the fact that protein represents at most 10% of the polysaccharide-protein complex (12).

Effect of pretreatment on the development of cellular immunity demonstrable in vitro. To determine whether GP-induced suppression could also be demonstrated in vitro, lymph node cells from mice pretreated with GP once or twice before immunization were stimulated with three antigen preparations derived from *C. albicans* (B-HEX, SCS, and GP) and the mitogens PHA, ConA, and PWM 12 days after the second cutaneous inoculation. The concentrations of antigen or mitogen which gave maximum values of stimulation were chosen for comparison and are presented in Table 1. Because culture conditions may vary slightly between experiments, data from two experiments are presented separately, each representing the results from triplicate cultures of lymph node cells pooled from three mice per group. Values given for B-HEX, SCS, and GP represent those obtained with 20, 100, and 200 μg per well, respectively, in experiment 1 and 10 μg and 200 μg per well for B-HEX and GP, respectively, in experiment 2. In experiment 1, maximum stimulation by mitogens was obtained with 1:200 and 1:100 dilutions from stock of PHA and PWM, respectively, and 1.25 μg of ConA per well, while in experiment 2, maximum values were obtained from PHA at 1:100 and ConA at 0.5 μg per well. Blastogenic responses to PHA and ConA were generally high, although cells from unpre-

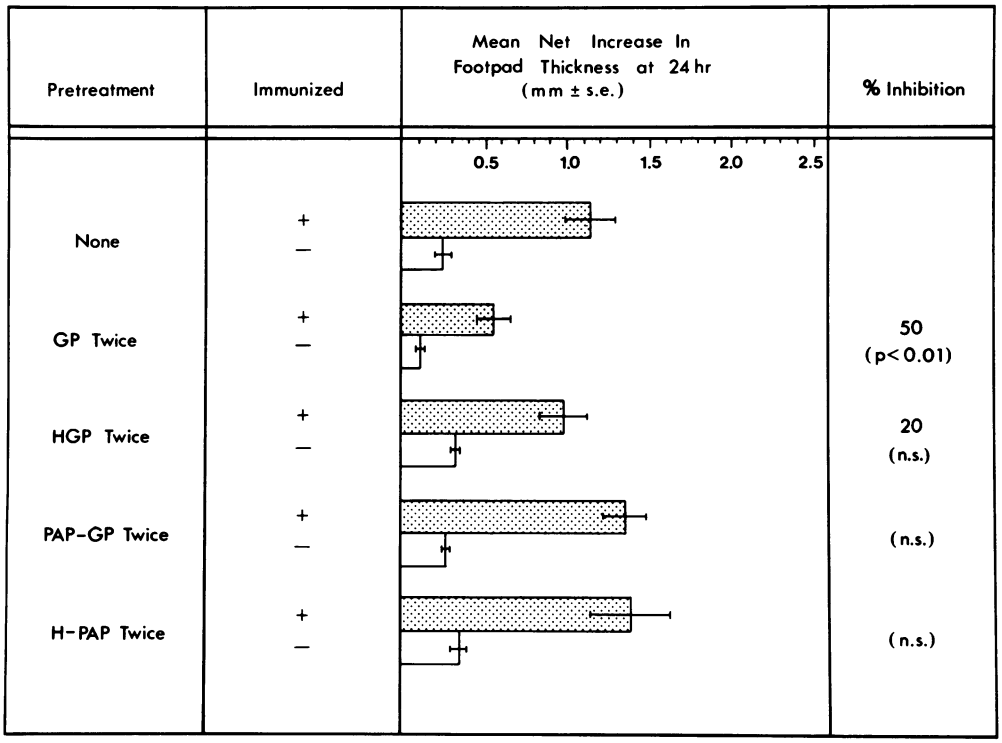

FIG. 3. Footpad responses to 30 μg of GP in immunized (stippled bars) and unimmunized (open bars) BALB/c mice pretreated twice before immunization. Pretreated mice received intravenous injections of 100 μg of GP, 100 μg of heat-treated GP (HGP), 200 μg of papain-treated GP (PAP-GP), or 100 μg of heat-treated papain (H-PAP). Percent inhibition = (increase in thickness for immunized unpretreated mice−increase for immunized pretreated mice)/(increase for immunized unpretreated mice) × 100. n.s., No significant difference. $n = 7$.

treated unimmunized controls were less responsive. The lower overall responses to PWM were probably a reflection of the smaller percentage of B cells found in lymph nodes. In general, for the mitogen-stimulated cultures there was no apparent suppressive effect of GP pretreatment in either immunized or unimmunized animals. In fact, ConA stimulation was highest in the pretreated immunized groups. Cells from pretreated and unpretreated immunized animals also responded well to B-HEX and SCS antigens, and as expected, there was no antigen stimulation in unimmunized groups. To the contrary, although GP pretreatment had no effect on the responses to B-HEX and SCS, suppression was observed in GP-stimulated cultures from both the twice-pretreated and once-pretreated immunized groups in experiment 1 and the group pretreated with GP 2 weeks before immunization in experiment 2.

Susceptibility of mice to intravenous challenge. Although pretreatment of mice with MAN or HKC did not result in the same alterations of cellular immunity as those demonstrated for GP, it was nevertheless of interest to determine whether such pretreatments might affect the protective response, which undoubtedly comprises several immune mechanisms. Two weeks after the second cutaneous inoculation, mice that were pretreated twice with GP, MAN, or HKC were challenged intravenously with 10^4 viable *C. albicans* blastoconidia. Four weeks later, animals were sacrificed for quantitative culture of kidneys and brains. Data from two representative experiments are presented in Fig. 4 and 5. In agreement with previous studies with CBA/J mice (16, 17, 25), the number of fungi cultured from brains and kidneys of unpretreated immunized mice was dramatically lower than that of unimmunized mice. The protective effect of immunization demonstrable in kidneys was not influenced adversely in a reproducible manner by any of the pretreatment regimens, although in two of four experiments (Fig. 4) GP pretreatment suppressed the protection in kidneys usually conferred by immunization, while HKC pretreatment, on the other hand, seemed to enhance protection in that organ (Fig. 5). Brains of mice pretreated with GP were uniformly more susceptible to infection, as demonstrated by four separate experiments in which reduction of CFU as a consequence of immunization was either insignificant (Fig. 5) or at best marginal (Fig. 4) in GP-pretreated animals. Moreover, CFU levels in the brains of GP-pretreated immunized animals were repeatedly higher than those of the unpretreated immunized group. Neither MAN nor HKC had such an effect on the protective response in the brain.

Antibody levels. To examine the effects of pretreatment with GP, MAN, or HKC on the development of humoral immune responses, blood was collected from each mouse at selected intervals before and after immunization and challenge for determination of anti-*Candida* antibody by enzyme-linked immunosorbent assay. The results for the detection of antibody directed against SCS or GP are shown in Fig. 6. The background levels of anti-*Candida* antibody in unmanipulated mice were low but detectable by this technique (Fig. 6A) and may have resulted from natural endogenous colonization of the gut or from stimulation by cross-reacting antigens. Five days after a second pretreatment with GP but not after pretreatment with MAN or HKC, both anti-SCS and anti-GP titers were boosted although not consistently (Fig. 6A). Immunization elicited an antibody response to SCS regardless of pretreatment, whereas it only induced anti-GP antibody in mice pretreated with GP or

TABLE 1. In vitro proliferative responses to mitogens and *C. albicans* antigens by lymph node cells of BALB/c mice[a]

Pretreatment[c]	Immunization[d]	Mean net cpm[b]					
		Mitogen added			Antigen added		
		PHA	ConA	PWM	GP	B-HEX	SCS
Expt 1							
None	+	59,079 (53,964–65,039)	63,811 (56,867–74,629)	33,431 (31,187–35,204)	25,471 (24,049–27,305)	30,055 (26,180–32,208)	42,360 (43,901–58,782)
None	−	35,558 (35,260–35,857)	38,045 (33,185–42,475)	17,296 (13.926–20,320)	<200	<200	<200
GP twice	+	63,551 (59,583–66,393)	85,801 (84,764–86,342)	35,843 (32,447–39,073)	10,376 (6,894–14,400)	29,003 (27,210–31,874)	46,506 (46,254–46,692)
GP once	+	64,240 (55,721–70,905)	80,615 (63,831–93,418)	28,683 (27,769–29,626)	17,137 (13,765–23,037)	23,188 (20,923–25,207)	46,665 (44,526–49,096)
GP twice	−	61,578 (56,912–68,354)	67,508 (59,324–81,200)	21,424 (19,097–23,350)	<200	<200	<200
Expt 2							
None	+	28,318 (25,351–31,758)	22,364 (17,031–27,426)		2,878 (2,570–3,106)	2,322 (2,309–2,335)	
None	−	18,927 (15,896–20,896)	13,449 (9,645–18,622)		<200	<200	
GP-2	+	23,526 (21,571–25,808)	30,848 (29,762–31,600)		1,582 (544–2,500)	2,635 (2,418–2,793)	
GP-2	−	24,522 (22,038–25,804)	33,473 (26,791–42,757)		<200	<200	
GP-1	+	18,374 (16,076–20,288)	21,278 (16,926–27,201)		2,236 (2,142–2,355)	2,050 (1,644–2,381)	
GP-1	−	15,581 (14,742–16,114)	19,731 (6,776–12,002)		<200	<200	

[a] Cultures of pooled cells from three mice per experimental group.
[b] Mean counts per minute of triplicate cultures of treated cells minus those of control cells. Range of values for each group is in parentheses.
[c] In experiment 1, pretreated mice received 100 μg of GP intravenously 1 week (GP once) or 1 and 2 weeks (GP twice) before immunization. In experiment 2, pretreated mice received 100 μg of GP intravenously 2 weeks (GP-2) or 1 week (GP-1) before immunization.
[d] +, Immunized; −, unimmunized.

MAN (Fig. 6B). While HKC pretreatment had a marginal effect on the production of anti-GP antibody in immunized mice, it considerably enhanced the anti-SCS response (Fig. 6B). There was a substantial increase in anti-SCS titers after intravenous challenge in both immunized and unimmunized mice, although the latter group did not attain as high levels as the former (Fig. 6C). Antibody to GP was not as markedly boosted by challenge. In fact, the highest titers to GP were attained in pretreated mice, regardless of whether they had been immunized (Fig. 6C). In general, pretreatment with any of the preparations had a selective effect on the antibody response, and when detected, it was immunopotentiating, even in mice in which the suppression of cellular immune responses could be demonstrated during the same period of observation.

DISCUSSION

This study was designed to assess the immunoregulatory potential of whole killed *C. albicans* blastoconidia or purified cell wall components administered to mice intravenously before an established immunization protocol. Since *Candida* antigenemia has now been recognized as an important feature of systemic infection, it was reasoned that an investigation of the effects of antigen on specific immune responses might help to explain some of the abnormalities seen clinically. The results indicate that at least one component of *C. albicans* cell walls, the GP, suppressed normal cellular immune responses to itself while at the same time potentiating antibody production, both activities in response to infection with *C. albicans*. Furthermore, the protective response was also impaired by pretreatment with GP, suggesting a greater importance of cellular rather than humoral mechanisms in the development of acquired immunity to *C. albicans*.

Suppression of cellular immune responses was demonstrable both in vivo and in vitro. DTH to GP was significantly inhibited in immunized animals that were pretreated once or twice with GP before immunization. The suppressive effect was highly antigen specific, however, since GP pretreatment did not alter the DTH responses of immunized mice to a different *Candida* antigen, B-HEX. In addition, GP pretreatment alone did not elicit a measurable DTH response in unimmunized animals. The in vitro manifestation of suppression was at the level of lymphocyte blastogenesis. Here again, however, it was specific for GP. Lymphoproliferative responses to the mitogens ConA, PHA, and PWM and the *Candida* antigens B-HEX and SCS were not influenced by GP pretreatment. As observed in vivo, GP pretreatment in the absence of immunization did not result in cellular responsiveness to any of the *Candida* antigens tested in vitro.

Because demonstration of DTH and *Candida*-specific lymphocyte proliferation correlated with the development of protective immunity in our model of candidiasis (16, 26), it was therefore highly significant that in this study, pretreated groups manifesting suppression of both measures of cell-mediated immunity were also more susceptible to systemic challenge. Protection in the kidneys of GP-pretreated immunized mice was uneven, but in the brain there was consistenly more colonization than in unpretreated immunized controls. A comparison of immunized and unimmunized groups revealed that GP pretreatment either abol-

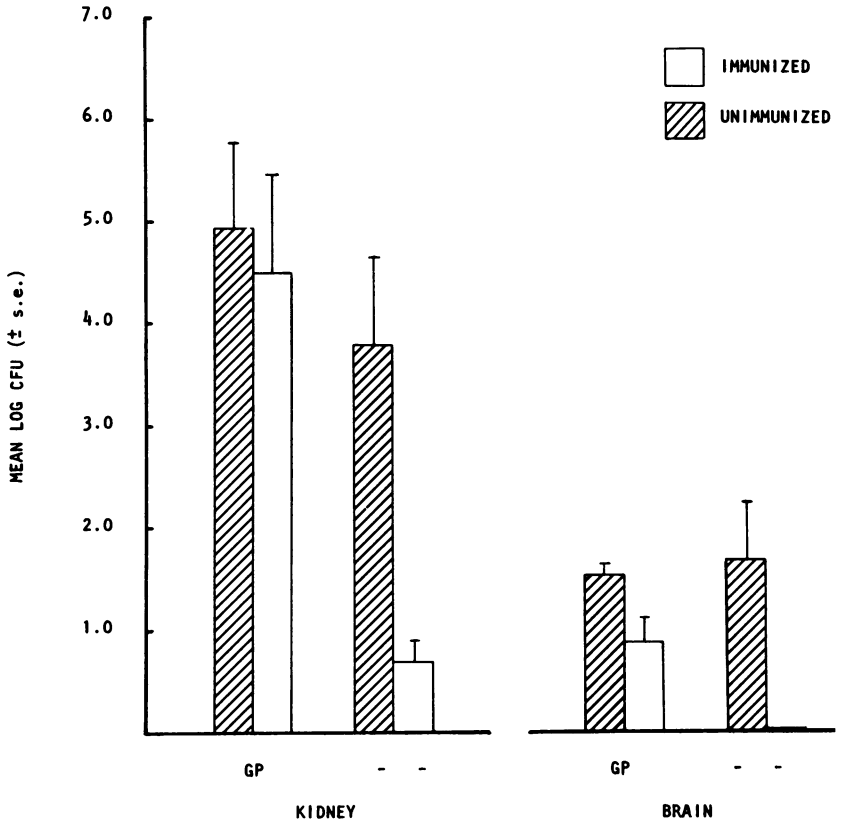

FIG. 4. Mean CFU in kidneys and brains of immunized and unimmunized BALB/c mice 4 weeks after intravenous challenge with 10^4 viable *C. albicans* blastoconidia. Pretreated mice received 100 μg of GP intravenously twice before immunization. $n = 8$. –, Unpretreated mice.

ished or greatly reduced the level of protection demonstrable in the brain normally conferred by immunization. Similarly, immunization of GP-pretreated mice did not always confer significant protection in kidneys, although in 50% of the experiments protection was normal in the kidneys of these mice. Thus, GP pretreatment blocked the development of protective immunity to the extent that significant colonization of the brain, and in many cases the kidneys, occurred despite the administration of an immunizing protocol.

The effect of GP pretreatment on in vivo antibody production was less remarkable, but a few interesting features were noted. As we had previously found in several strains of mice (6, 19), immunization with two cutaneous injections of viable *C. albicans* induced moderate production of antibody directed against SCS, while intravenous challenge had a similar effect in naive mice and boosted titers in immunized mice. Pretreatment with any of the antigen preparations, GP, MAN, or especially HKC, enhanced production of anti-SCS antibody in immunized mice. In the absence of immunization, both GP and HKC but not MAN elicited some anti-SCS antibody. Generation of antibody directed against GP in response to immunization or intravenous challenge was negligible, while GP itself was somewhat antigenic. When animals were pretreated with GP or MAN, however, higher titers to GP were obtained after immunization compared with those from unpretreated immunized controls. Interestingly, MAN pretreatment in the absence of subsequent immunization resulted in a somewhat delayed development of anti-GP antibody. Thus, not only did GP and MAN pretreatment boost anti-GP titers after immunization, but also antibody directed against GP could be generated as a consequence of either GP or MAN inoculation alone, suggesting a degree of cross-reactivity between the two antigens. This observation is not surprising, since MAN comprises most of the carbohydrate present in GP (12, 23). It is unclear why cross-reactivity between the two cell wall preparations was observed at the humoral but not the cellular level, although it may be a reflection of the differential stimulation (or suppression) of antibody or cell-mediated responses by carbohydrate and protein antigens. We find the dual effect of GP, i.e., normal or enhanced humoral responses concomitant with depressed cellular responses, to be particularly interesting, since clinically it is not uncommon to find normal or even high titers of specific antibody in patients manifesting decreased cellular responsiveness.

The importance of the protein moiety of GP in eliciting DTH responses was established previously when it was determined that protease treatment entirely abrogated the ability of GP to elicit a DTH response in immunized mice (12). Likewise, we demonstrated in this study that protease treatment, and to a lesser extent heat treatment, removed the immunosuppressive potential of GP as well. Thus, in contrast to other studies in which several groups have demonstrated immunosuppression at the level of purified polysaccharide, e.g., pneumococcal (42) or cryptococcal (27) polysaccharide antigens, we found the immunosuppressive potential of a *Candida* cell wall antigen to reside in the heat-labile, protease-sensitive portion of the polysaccharide-protein complex.

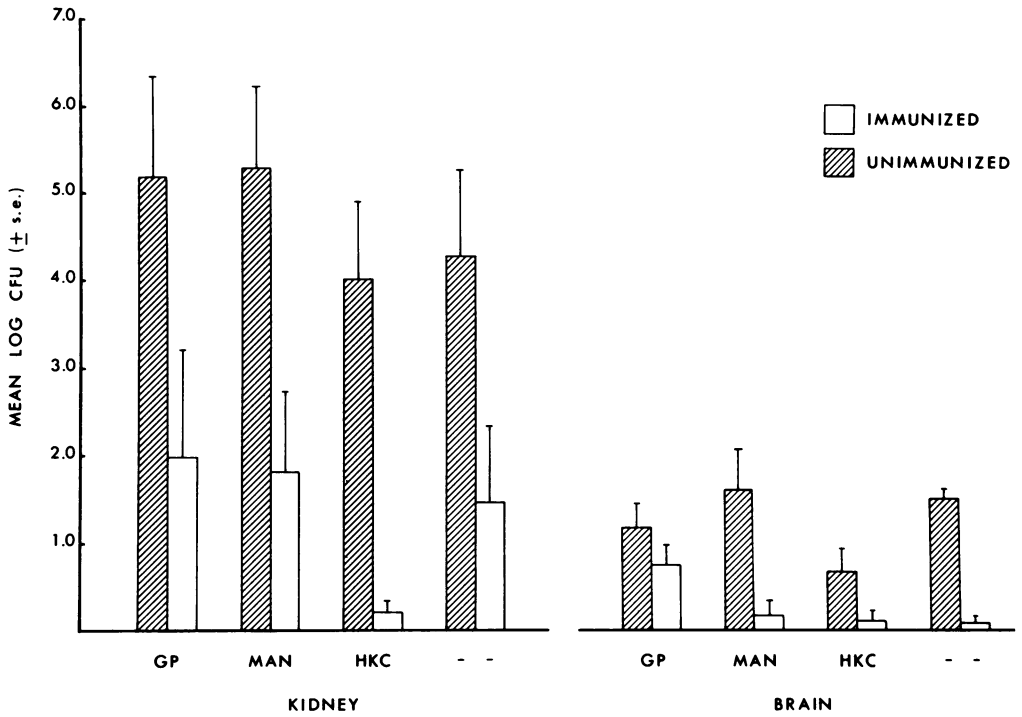

FIG. 5. Mean CFU in kidneys and brains of immunized and unimmunized BALB/c mice 4 weeks after intravenous challenge with 10^4 viable *C. albicans* blastoconidia. Pretreated mice received 100 μg of GP, 100 μg of MAN, or 5×10^7 HKC intravenously twice before immunization. $n = 8$. –, Unpretreated mice.

Despite the fact that MAN antigenemia has been documented in candidiasis (10) and has been implicated in suppression of lymphocyte proliferation in some patients with chronic mucocutaneous candidiasis (13), purified MAN as used in the current studies had no such suppressive effect when administered in vivo. Others have demonstrated MAN-induced suppression of both innate and specific cell-mediated responses in vitro as well (14, 28, 44). In one study, it was suggested that free MAN may inhibit myeloperoxidase-mediated candidacidal activity of neutrophils by binding the enzyme before it can bind to target yeast cells (44). There is also evidence that suppression of cellular immune responses in patients with chronic mucocutaneous candidiasis is due to the interference by circulating MAN of monocyte-mediated antigen presentation to T lymphocytes (14). We cannot rule out the possibility that such a mechanism exists in the murine system, since our failure to detect MAN-induced suppression in vivo could be due to the relatively low dose used for pretreatments and the fact that we assayed for immune responses at a time when circulating MAN had presumably been cleared by the reticuloendothelial system.

It is also unlikely that the mechanism of GP-induced suppression reported here is one of direct interference with antigen handling, for the same reasons outlined for MAN. The suppressive mechanism may be due to the induction of a specific suppressor cell population, such as that described for experimental histoplasmosis (9), or it may, on the other hand, be a largely nonspecific effect due to toxicity exerted by GP, possibly in connection with the reported pharmacological effects of GP extracts of *Candida* cell walls, i.e., mast cell degranulation and histamine release (30, 41). The administration of yeast cell wall GP preparations reportedly induces a shocklike state in laboratory animals, similar to that observed with bacterial endotoxins (8, 20, 29, 41) and resembling the shock characteristically found in the septic state of acute disseminated candidiasis (5, 20). Indeed, some of the pathophysiological effects of GP administration can be explained on the basis of histamine release (31). There is a growing body of evidence supporting a modulatory role for histamine in various immune responses, apparently via activation of T-suppressor cells bearing H-2 receptors (3, 4, 18). Thus, it is possible that GP pretreatment causes the release of enough histamine to activate a subpopulation of suppressor cells that blocks the development of a complete immune response to subsequent infection with viable *C. albicans*. Studies directed towards the identification and characterization of such a suppressor cell population, particularly with regard to the presence of H-2 receptors, seem warranted. In addition, T-suppressor cell activity has been observed in human lymphocyte cultures stimulated by a glucomannan-protein preparation of *C. albicans* (33).

Intravenous inoculation with viable or nonviable whole *C. albicans* blastoconidia has been reported to induce transient nonspecific (35–38, 43) or specific (39) suppression of immune responses in experimental animals. In the study described here, we found no evidence of suppression of immunity as a consequence of pretreatment with a large dose of HKC. To the contrary, HKC pretreatment potentiated antibody responses to SCS antigen and enhanced protection against intravenous challenge demonstrable in kidneys of immunized mice. The lack of agreement between our results and those reported previously probably reflects a difference in experimental design. The studies of Segal and co-workers (38, 43), Skerl et al. (39), and Rogers and co-workers (35–37) all share a common approach in that they investigated the effects of blastoconidia preparations systemically administered to animals not previously sensitized to *C. albicans*. Thus, for the most part, they provide information about innate responses to infection, whereas our studies were oriented towards acquired immunity. The *Candida*-induced

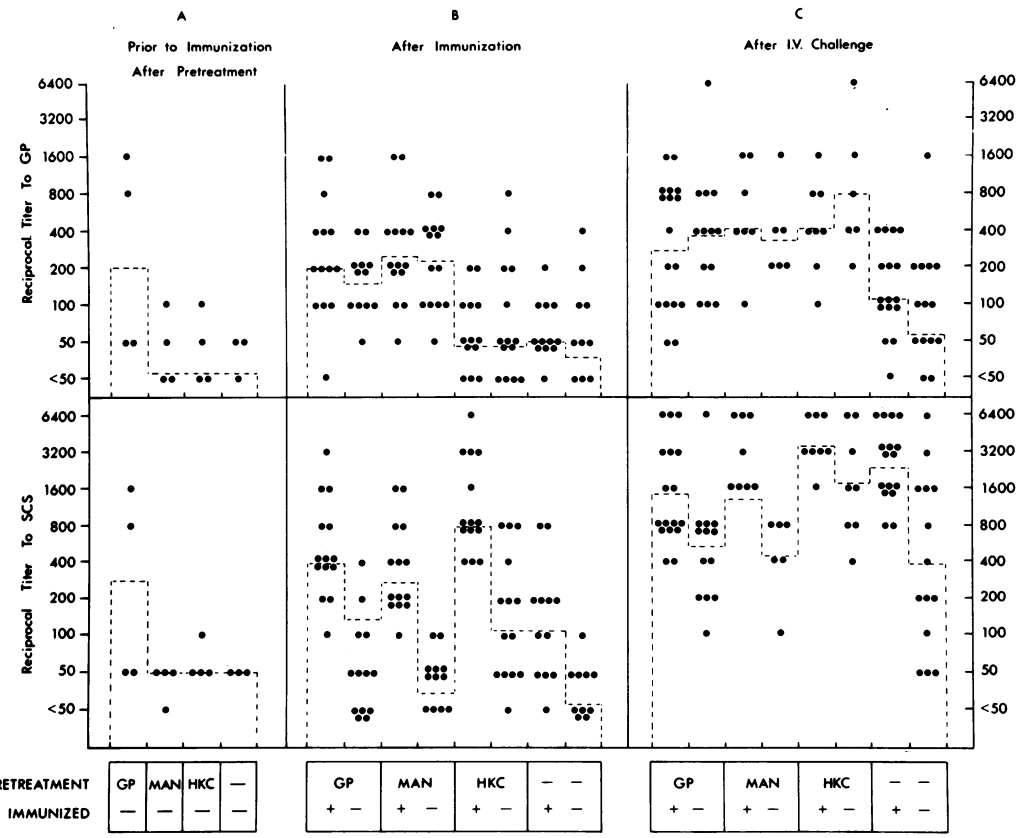

FIG. 6. Antibody titers (immunoglobulin G plus immunoglobulin M) to SCS or GP as determined by enzyme-linked immunosorbent assay in immunized and unimmunized BALB/c mice 5 days after second pretreatment (A), 9 days after second cutaneous inoculation (B), and 28 days after intravenous challenge with viable *C. albicans* (C). Pretreated mice received 100 μg of either GP or MAN or 5×10^7 HKC intravenously twice before immunization. Geometric mean values are represented by the dashed lines and were calculated by converting titers to log values before averaging, with titers of <50 assigned a value of zero.

suppressor cell population described by Rivas and Rogers (35) may be part of a regulatory pathway entirely separate from the mechanism responsible for the immunosuppression we have reported here. Future experiments designed to confirm the existence of a suppressor cell population in our model should serve to clarify the discrepancy.

In summary, an immunosuppressive potential for the cell wall GP of *C. albicans* was demonstrated, the features of which differ somewhat from those of *Candida*-induced immunosuppression reported elsewhere. Pretreatment of mice with sublethal doses of GP resulted in prolonged and specific suppression of cellular immune responses and a significant impairment of the development of protective immunity, while at the same time potentiating humoral responses. The suppressive mechanism is undefined at this time but may have to do with the histamine-releasing properties of the GP.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants AI-12806 and AI-07152 from the National Institute of Allergy and Infectious Diseases.

We thank Frank Lyon and Karin Lowe for technical assistance and Geraldine Brooks for assistance in preparation of the manuscript. E.W.C. was a trainee in medical mycology.

LITERATURE CITED

1. **Alford, R., and R. A. Goodwin.** 1973. Variation in lymphocyte reactivity to histoplasmin during the course of chronic pulmonary histoplasmosis. Am. Rev. Respir. Dis. **108**:85–91.
2. **Aronson, I. K., C. H. L. Rieger, K. Soltani, V. Tkalcevic, W. C. Chan, A. L. Lorincz, and G. Matz.** 1979. Late onset chronic mucocutaneous candidiasis with lymphoma and specific serum inhibitory factor. Cancer **43**:101–108.
3. **Askenase, P. W., A. Schwartz, J. N. Siegel, and R. K. Gershon.** 1981. Role of histamine in the regulation of cell-mediated immunity. Int. Arch. Allergy Appl. Immunol. **66**(Suppl. 1):225–233.
4. **Beer, D. J., and R. E. Rocklin.** 1984. Histamine-induced suppressor-cell activity. J. Allergy Clin. Immunol. **73**:439–452.
5. **Braude, A. I., and J. A. Rock.** 1959. The syndrome of acute disseminated moniliasis in adults. Arch. Intern. Med. **104**: 107–116.
6. **Carrow, E. W., R. F. Hector, and J. E. Domer.** 1984. Immunodeficient CBA/N mice respond effectively to *Candida albicans*. Clin. Immunol. Immunopathol. **33**:371–380.
7. **Catanzaro, A., L. Spitler, and K. M. Moser.** 1974. Immunotherapy of coccidioidomycosis. J. Clin. Invest. **54**: 690–701.
8. **Cutler, J. E., L. Friedman, and K. C. Milner.** 1972. Biological and chemical characterization of toxic substances from *Candida albicans*. Infect. Immun. **6**:616–627.
9. **Deepe, G. S., Jr., S. R. Watson, and W. E. Bullock.** 1982. Generation of disparate immunoregulatory factors in two inbred strains of mice with disseminated histoplasmosis. J. Immunol. **129**:2186–2191.
10. **de Repentigny, L., and E. Reiss.** 1984. Current trends in immunodiagnosis of candidiasis and aspergillosis. Rev. Infect. Dis. **6**:301–312.
11. **Diamond, R. D., and J. E. Bennett.** 1973. Disseminated

cryptococcosis in man. Decreased lymphocyte transformation in response to *Cryptococcus neoformans*. J. Infect. Dis. **127**:694–697.
12. **Domer, J. E., and S. A. Moser.** 1978. Experimental murine candidiasis: cell-mediated immunity after cutaneous challenge. Infect. Immun. **20**:88–98.
13. **Fischer, A., J. J. Ballet, and C. Griscelli.** 1978. Specific inhibition of *in vitro Candida*-induced lymphocyte proliferation by polysaccharidic antigens present in serum of patients with chronic mucocutaneous candidiasis. J. Clin. Invest. **62**: 1005–1013.
14. **Fischer, A., L. Pichat, M. Audinot, and C. Griscelli.** 1982. Defective handling of mannan by monocytes in patients with chronic mucocutaneous candidiasis resulting in a specific cellular unresponsiveness. Clin. Exp. Immunol. **47**:653–660.
15. **Gatenby, P., A. Basten, and E. Adams.** 1980. Thymoma and late onset mucocutaneous candidiasis associated with a plasma inhibitor of cell mediated immune function. J. Clin. Lab. Immunol. **3**:209–216.
16. **Giger, D. K., J. E. Domer, and J. T. McQuitty, Jr.** 1978. Experimental murine candidiasis: pathological and immune responses to cutaneous inoculation with *Candida albicans*. Infect. Immun. **19**:499–509.
17. **Giger, D. K., J. E. Domer, S. A. Moser, and J. T. McQuitty, Jr.** 1978. Experimental murine candidiasis: pathological and immune responses in T-lymphocyte-depleted mice. Infect. Immun. **21**:729–737.
18. **Hebert, J., R. Beaudoin, M. Aubin, and M. Fontaine.** 1980. The regulatory effect of histamine on the immune response: characterization of the cells involved. Cell. Immunol. **54**:49–57.
19. **Hector, R. F., J. E. Domer, and E. W. Carrow.** 1982. Immune responses to *Candida albicans* in genetically distinct mice. Infect. Immun. **38**:1020–1028.
20. **Kettner, M., R. Para, and T. Trnovec.** 1983. Hepatic and plasma lysosomal enzyme activity in shock-like state following administration of polysaccharide-protein complex isolated from *Candida albicans*. Circ. Shock **10**:31–39.
21. **Kirkpatrick, C. H., R. R. Rich, and J. E. Bennett.** 1971. Chronic mucocutaneous candidiasis: model-building in cellular immunity. Ann. Intern. Med. **74**:955–978.
22. **Kocourek, J., and C. E. Ballou.** 1969. Method for fingerprinting yeast cell wall mannans. J. Bacteriol. **100**:1175–1181.
23. **Korn, E. D., and D. H. Northcote.** 1960. Physical and chemical properties of polysaccharides and glycoproteins of the yeast-cell wall. Biochem. J. **75**:12–17.
24. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
25. **Moser, S. A., and J. E. Domer.** 1980. Effects of cyclophosphamide on murine candidiasis. Infect. Immun. **27**: 376–386.
26. **Moser, S. A., J. E. Domer, and F. J. Mather.** 1980. Experimental murine candidiasis: cell-mediated immunity after cutaneous challenge. Infect. Immun. **27**:140–149.
27. **Murphy, J. W., and J. W. Moorhead.** 1982. Regulation of cell-mediated immunity in cryptococcosis. I. Induction of specific afferent T suppressor cells by cryptococcal antigen. J. Immunol. **128**:276–283.
28. **Nelson, R. D., M. J. Herron, R. T. McCormack, and R. C. Gehrz.** 1984. Two mechanisms of inhibition of human lymphocyte proliferation by soluble yeast mannan polysaccharide. Infect. Immun. **43**:1041–1046.
29. **Nosál, R., V. Nosálová, and D. Šikl.** 1979. Hemodynamic effects of the glycoprotein isolated from cell wall of *Candida albicans*. Toxicon **17**:668–672.
30. **Nosál, R., J. Novotný, and D. Šikl.** 1974. The effect of glycoprotein from *Candida albicans* on isolated rat mast cells. Toxicon **12**:103–108.
31. **Nosálová, V., T. Trnovec, M. Greguškova, and R. Nosál.** 1979. The effect of polysaccharide-protein complex isolated from *Candida albicans* on regional blood flow in rats. Experientia **35**:341–342.
32. **Peat, S., W. J. Whelan, and T. E. Edwards.** 1961. Polysaccharide of baker's yeast. Part IV. Mannan. J. Chem. Soc. **1961**:29–34.
33. **Piccolella, E., G. Lombardi, and R. Morelli.** 1981. Generation of suppressor cells in the response of human lymphocytes to a polysaccharide from *Candida albicans*. J. Immunol. **126**: 2151–2155.
34. **Restrepo-Moreno, A., and J. D. Schneidau, Jr.** 1967. Nature of the skin-reactive principle in culture filtrates prepared from *Paracoccidiodes brasiliensis*. J. Bacteriol. **93**:1741–1748.
35. **Rivas, V., and T. J. Rogers.** 1983. Studies on the cellular nature of *Candida albicans*-induced suppression. J. Immunol. **130**: 376–379.
36. **Rogers, T. J., and E. Balish.** 1978. Effect of systemic candidiasis on blastogenesis of lymphocytes from germfree and conventional rats. Infect. Immun. **20**:142–150.
37. **Rogers, T. J., and E. Balish.** 1978. Suppression of lymphocyte blastogenesis by *Candida albicans*. Clin. Immunol. Immunopathol. **10**:298–305.
38. **Segal, E., H. Sandovsky-Losica, and N. Vardinon.** 1980. Suppressive action of cytoplasmic and metabolic extracts of *Candida albicans* on the immune response in guinea pigs. Mycopathologia **72**:121–128.
39. **Skerl, K. G., W. M. Scheld, G. M. Alliegro, and R. A. Calderone.** 1980. Lymphocyte blastogenesis during experimental endocarditis caused by *Candida albicans*. J. Reticuloendothel. Soc. **28**:495–506.
40. **Stobo, J. D., S. Paul, R. E. van Scoy, and P. E. Hermans.** 1976. Suppressor thymus-derived lymphocytes in fungal infection. J. Clin. Invest. **57**:319–328.
41. **Švec, P.** 1974. On the mechanism of action of glycoprotein from *Candida albicans*. J. Hyg. Epidemiol. Microbiol. Immunol. **18**:373–376.
42. **Taylor, C. E., D. F. Amsbaugh, P. W. Stashak, G. Caldes, B. Prescott, and P. J. Baker.** 1983. Cell surface antigens and other characteristics of T cells regulating the antibody response to type III pneumococcal polysaccharide. J. Immunol. **130**:19–23.
43. **Vardinon, N., and E. Segal.** 1979. Suppressive action of *Candida albicans* on the immune response in mice. Exp. Cell Biol. **47**:275–280.
44. **Wright, C. D., J. U. Bowie, G. R. Gray, and R. D. Nelson.** 1983. Candidacidal activity of myeloperoxidase: mechanisms of inhibitory influence of soluble cell wall mannan. Infect. Immun. **42**:76–80.