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Mannan as an Antigen in Cell-Mediated Immunity (CMI) Assays and as a Modulator of Mannan-Specific CMI

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Mannan (MAN) extracted from *Candida albicans* 20A was investigated for its potential as an antigen in the detection of cell-mediated immunity (CMI) in vivo and in vitro and for its ability to modulate CMI when administered intravenously (i.v.). CBA/J mice were either immunized as adults by the cutaneous inoculation of 10^6 viable blastoconidia or colonized as infants (primed) and then boosted cutaneously as adults. When immunized animals were footpad tested with MAN, highly significant delayed-type hypersensitivity (DH) responses were detected. The DH responses to MAN were of a greater magnitude than those noted with the same quantity of cell wall glycoprotein (GP), an ethylenediamine extract of the cell wall which contains both glucan and MAN. In contrast, GP was a better antigen for the detection of CMI responses in an in vitro lymphoproliferative assay with either spleen or lymph node cell suspensions. Mice treated with MAN i.v. prior to the initiation of immunization or between priming and secondary inoculations developed significantly suppressed DH reactions when tested with either MAN or GP. The lowest effective dose of MAN was 250 μg, maximum suppression occurred with 500 μg, and either dose given 1 week prior to immunization was suppressive. The suppression by MAN was specific for MAN or the MAN-containing GP. Responses to another unrelated candidal antigen, a membrane extract designated BEX, were relatively unaffected. MAN, therefore, was an effective antigen for the detection of CMI in vivo, and its administration i.v. created what appeared to be a MAN-specific suppression since it could be detected with both MAN and a MAN-containing extract from the cell wall. Caution must be exercised in the interpretation of these data, however, since the protein component of each of these extracts has not been characterized with respect to its potential role in the phenomena observed.

Serum-dependent abnormalities occurring during episodes of candidiasis have been described on numerous occasions, especially in patients suffering from chronic mucocutaneous candidiasis (CMCC; 1, 5, 7, 16, 18, 28, 39, 49), but also in patients with disseminated (27) and recurrent vaginal candidiasis (50). The nature of the immunoinhibitor found in the serum of patients with candidiasis is not entirely clear, and in fact, existing evidence points to multiple etiologies for the phenomenon, ranging from a product of a thymoma (18) to candidal antigen (5, 16, 28, 39). Perhaps the most common suggested etiology, however, is circulating surface antigen, in particular, some form of the cell wall polysaccharide, mannan (MAN; 16, 39). Fischer et al. (16) isolated a polysaccharide from the serum of one of the CMCC patients they studied and determined that it was nondialyzable, thermostable, and nonprecipitable with ammonium sulfate and could be adsorbed from serum by anti-*Candida* antibodies and concanavalin A (ConA), all of which are compatible with the idea that the circulating inhibitor is MAN. Moreover, the inhibitor disappeared during antifungal therapy, and its inhibitory effects could be reproduced by adding purified MAN from *Candida albicans* to cultures of lymphocytes from healthy subjects.

Aside from the serum-dependent abnormalities noted in vitro at the level of phagocytosis or other activities associated with polymorphonuclear leukocyte function, e.g., chemotaxis or killing ability (25, 48, 49, 51), the most frequently described abnormality has been associated with cell-mediated immunity (CMI) phenomena. In particular, delayed-type hypersensitivity (DH) is usually negative, and lymphocytes from patients respond poorly to mitogens and candidal antigens in in vitro culture. Although MAN has been implicated as the causative agent in many instances of CMI dysfunction, it has not been the antigen used to measure those suppressed responses. In fact, it has not yet been established definitively that MAN is a suitable antigen for the detection of CMI phenomena, despite the fact that it is clearly immunogenic in humans for the induction of MAN-specific antibody production. There are only a few studies in which MAN was used to detect CMI (2, 15, 19, 44, 46). Two groups of investigators (2, 19) examined MAN as an antigen in the lymphocyte stimulation assay, finding minimal responses at best, and three groups of investigators (15, 44, 46) examined MAN as an antigen in vivo in experimental animals, viz., guinea pigs. Infected animals were used in only one of those studies, and MAN elicited the poorest responses of the antigens tested.

We have been studying the potential for components of the candidal cell wall, viz., cell wall glycoprotein (GP) and MAN, to regulate immune responses in mice (6, 12). Studies reported by us previously with MAN, however, were limited to examinations of immune responses to antigens unrelated to *C. albicans* (12). In view of the paucity of information regarding MAN as a suitable antigen for the detection of MAN-specific CMI, we initiated studies to determine whether MAN-specific responses could be detected in an experimental murine model of candidiasis. Since MAN-specific responses were detected, a study of the potential for in vivo MAN treatment to alter MAN-specific responses, as well as immune responses unrelated to MAN itself, was

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initiated. In this paper, therefore, MAN-specific CMI responses are described as well as MAN-induced suppression.

MATERIALS AND METHODS

Mice. Male and female CBA/J mice, 6 to 8 weeks of age, referred to hereafter as adults, were obtained from Jackson Laboratory, Bar Harbor, Maine, for use in specific experiments or as animals for mating in order to obtain infant mice in which long-term colonization with *C. albicans* could be established as described previously (10, 43). Specific mating and colonization procedures have been described previously (10). All animals were housed under laminar flow conditions with negative-pressure air flow and fed mouse chow and water ad libitum.

Culture methods and fractionation procedures. *C. albicans* 20A, a serotype A isolate originally obtained from Errol Reiss (Centers for Disease Control, Atlanta, Ga.) was used throughout these studies. It was maintained at 4°C by monthly transfer on Sabouraud dextrose agar. Viable blastoconidia for inoculation into mice were grown in Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy dialysate broth (45) for 18 h at 37°C on a gyratory shaker operating at approximately 165 rpm. The blastoconidia were washed three times in sterile nonpyrogenic saline, counted, and resuspended to the desired concentration in the same diluent for inoculation into animals. Each suspension was diluted further and spread onto Sabouraud agar plates to determine viable numbers. Viable blastoconidia from which antigens were to be prepared were harvested from 10-liter fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) cultures incubated for 18 h at 37°C with constant aeration and agitation and washed with 0.15 M phosphate-buffered saline (PBS; pH 7.2) containing 0.001 M phenylmethylsulfonyl fluoride (PBS-inhibitor). Blastoconidia were stored frozen in PBS-inhibitor before fractionation.

MAN was extracted from whole blastoconidia by the method of Peat et al. (40) as modified by Kocourek and Ballou (26). It was dialyzed against distilled water and lyophilized. Each extract prepared contained 6 to 8% protein as determined by the method of Lowry et al. (30). Other subcellular components of *C. albicans*, e.g. GP, soluble cytoplasmic substances (SCS), and a membrane extract (BEX), were prepared as previously described (10, 11, 33). Briefly, GP was obtained from isolated cell walls by ethylenediamine extraction following breakage of cells by ballistic action with a Bead Beater (Biospec Products, Bartlesville, Okla.), and it was dialyzed for 4 days against 0.4 M sodium chloride to remove the ethylenediamine prior to dialysis in water. BEX was obtained from the membrane fraction of broken cells by extraction of butanol-extracted membranes with hot PBS, and SCS contained soluble cytoplasmic components remaining in the supernatant of a broken-cell suspension centrifuged at 100,000 × *g*.

Animal inoculations. Infant mice were infected with 10^7 viable *C. albicans* blastoconidia by intubation at 5 to 6 days of age by using small-bore-diameter polyethylene tubing as described previously (10, 43). Confirmation of colonization was obtained by fecal culture of intubated animals when they were 4 to 5 weeks of age. Other animals were never intubated but were inoculated cutaneously on the shaved flank as adults with 10^6 viable *C. albicans* blastoconidia to stimulate immunity (20).

Footpad testing. DH responses were determined by footpad testing with 0.02 ml of nonpyrogenic saline (NPS) containing the appropriate concentrations of antigen, viz., 30 μg (wt/vol) GP or MAN and 20 μg of BEX (weight of protein per milliliter). Footpads were measured with Schnelltaster calipers (H. Kroplein, Schluchtern, Federal Republic of Germany) before injection of antigen and at 15 min and at 4, 7, 24, and 48 h after injection. The calipers are capable of measuring increments of 0.05 mm in thickness. The mean net increase in footpad thickness was determined by subtracting the preinjection measurements from the postinjection measurements. Unimmunized animals were used as controls. Animals were never tested twice or with two antigens. Data were analyzed by the Student *t* test.

Lymphocyte stimulation. Proliferative responses of splenocytes or cells from inguinal lymph nodes draining the site of cutaneous inoculation were assayed by a micromethod described previously (21). The mitogens used were phytohemagglutinin P (Difco Laboratories, Detroit, Mich.) at 1:25, 1:50, and 1:100 dilutions and ConA (Sigma Chemical Co., St. Louis, Mo.) at 0.4, 2.0, and 10.0 μg per well. The antigens used were GP and MAN at 100 and 200 μg (wt/vol) per well, BEX at 10 and 20 μg of protein per well, and SCS at 25 and 50 μg (wt/vol) per well. Mitogen-stimulated cultures were harvested after 72 h of incubation, and antigens were harvested after 96 h, each having been pulsed with 0.5 μCi of [^{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. When appropriate, data were analyzed by the Student *t* test.

Experimental design. The manner in which the animals were immunized and the times selected at which the various immune parameters were measured were based on previous studies in the CBA/J mouse (10, 11, 20, 33). Animals were immunized in one of two ways. First, intubation of infant mice resulted in long-term colonization and immunization, the latter of which was demonstrated readily following a single cutaneous inoculation (booster) of mice with viable blastoconidia when the mice were >5 weeks of age (10). Second, adult animals were immunized by the cutaneous inoculation of viable *C. albicans* administered twice, two weeks apart, on opposite shaved flanks (20). Maximum DH responses occurred 1 week following the last of the two cutaneous inoculations in mice infected cutaneously only (11) and 1 week following the single cutaneous inoculation administered to intubated animals (10). We have shown previously that males and females develop similar levels of DH in response to immunization (10); thus both sexes were used, depending upon the availability of the animals. The sexes were not mixed within a given experiment, however. MAN was administered at various times prior to cutaneous challenge of colonized mice and prior to the first or second cutaneous inoculation in mice immunized by cutaneous infection only.

RESULTS

Demonstration of the antigenicity of MAN in vivo and in vitro. Adult, previously uninfected male mice were immunized by cutaneous inoculation with *C. albicans* and tested 1 week later for DH with 30 μg each of MAN or GP, the latter being an antigen which was used successfully previously (33). The 24-h footpad reactions measured in three separate experiments are presented in Table 1. In each experiment, the reactions to MAN were greater than the reactions to GP. In only two of the experiments were the data statistically significant, however. The temporal development of the footpad reaction for each antigen was similar, e.g., the 15-min,

TABLE 1. Ability of comparable amounts (30 μg [wt/vol]) of MAN and GP to elicit DH responses in unimmunized mice or mice immunized by the cutaneous route with viable *C. albicans* 20A

Expt[a]	Mean net increase in footpad thickness (mm ± SEM) at 24 h with:				P^d
	MAN		GP		
	IMM[b]	UNIMM[c]	IMM	UNIMM	
I	1.78 ± 0.15	0.06 ± 0.04	1.41 ± 0.09	0.09 ± 0.02	≤0.050
II	1.41 ± 0.07	0.09 ± 0.02	1.01 ± 0.28	0.03 ± 0.01	≤0.375
III	1.32 ± 0.13	0.11 ± 0.07	0.64 ± 0.18	0.09 ± 0.04	≤0.010

[a] In experiments I and III, CBA/J adult males were used; in experiment II, CBA/J adult females were used.
[b] IMM, Mice immunized by the cutaneous route with viable *C. albicans* 20A.
[c] UNIMM, Unimmunized mice.
[d] Statistical significance determined by the Student *t* test when MAN and GP were compared between immunized mice.

4-, 7-, 24-, and 48-h responses (mean ± standard error of the mean) for MAN were 0.32 ± 0.07, 0.72 ± 0.06, 1.18 ± 0.13, 1.78 ± 0.15, and 1.25 ± 0.17 mm, respectively, while those for GP were 0.24 ± 0.07, 0.16 ± 0.06, 0.61 ± 0.12, 1.41 ± 0.09, and 0.83 ± 0.10 mm, respectively. The primary difference between the two responses in immunized animals, other than the magnitudes of the 24- and 48-h responses, was that the 4-h response to GP was usually less than the 15-min response, whereas the 4-h response to MAN was usually greater than the 15-min response. When mice were tested with 10 μg of each antigen (data not shown), the animals tested with MAN still had greatly elevated responses, but those tested with GP developed reactions which were barely significantly different from control values. Although data are presented in Table 1 for mice immunized by the cutaneous route only, mice immunized by colonization as infants or adults and boosted by cutaneous inoculation also responded well to MAN as an antigen for the detection of DH (e.g., see Fig. 2).

GP and MAN, along with the membrane and cytoplasmic antigens, BEX and SCS, were compared for their ability to induce lymphoproliferative responses in vitro as well. In one experiment, CBA/J female mice which had been colonized as infants and then inoculated cutaneously with viable blastoconidia as adults, in this instance when about 12 weeks of age, were sacrificed for assay either 6 or 12 days following the cutaneous inoculation. In another experiment, similarly treated male CBA/J mice were sacrificed for assay 9 days following the cutaneous inoculation. Each group consisted of three mice. Lymph nodes draining the site of the cutaneous lesion, or comparable lymph nodes in uninfected mice, were pooled for testing, whereas two spleens from each group were tested independently at each interval in experiment I or pooled in experiment II. The data obtained are presented in Table 2. GP was clearly superior to MAN as an antigen for the detection of CMI in 6-, 9-, and 12-day assays, as evidenced from the proliferation observed with lymphocytes from colonized-cutaneously infected animals or animals inoculated cutaneously only. Moreover, the in vitro lymphocyte responses to BEX and SCS with cells from immunized animals were similar to those for GP in experiment I.

Mitogen responses were determined primarily to ascertain that the cell suspensions were healthy, but some generalizations can be made concerning the data acquired. The higher values noted for the lymph node cells in the colonized-cutaneously infected animals probably reflect a proportionately greater number of T lymphocytes in the lymph nodes of colonized animals undergoing a secondary response to the cutaneous challenge. Indeed, in another experiment (data not shown) involving CBA/J female mice, that same group had a greater proliferative response to ConA at 6 days also, but in addition, animals inoculated cutaneously only were more responsive to mitogen, as indicated for the male mice tested on day 9 (Table 2). In fact, in most groups, T cell mitogen responses of lymph node cells were directly proportional to antigen reactivity in the cell population, as might be expected, since antigen reactivity to BEX and SCS have been determined to be T-cell specific (33).

Spleen cell responses to antigen showed the same general pattern as did the lymph node cells, but the level of activity in the spleen was usually less than that of the draining lymph nodes. The relationships observed among the various groups for splenic mitogen responses, however, were different than those for lymph node lymphocytes. Splenocyte mitogen responses for infected animals were seldom elevated over those observed in control animals, and in fact, there was a tendency for the splenic lymphocyte response to both PHA and ConA in both males and females inoculated cutaneously with *C. albicans* to be suppressed compared with the responses of control animals.

Suppression of DH response to MAN by parenteral treatment with MAN. As demonstrated above, DH could be detected in mice infected with *C. albicans* by testing with MAN. It was of interest, therefore, to determine if the DH response could be altered by parenteral treatment with MAN prior to or during the immunization procedure. In the first of a series of experiments, mice were inoculated intravenously (i.v.) with two different concentrations of MAN, viz., 1,000 and 100 μg, on three occasions, beginning 2 weeks before the initiation of immunization. The immunization procedure involved two cutaneous inoculations with viable *C. albicans* 2 weeks apart, and DH was determined by footpad testing with 30 μg of MAN 1 week following the second cutaneous inoculation. As can be seen from the upper portion of Fig. 1, treatment with 1,000 μg of MAN induced suppression which was highly significant ($P \leq 0.0005$) regardless of the treatment regimen, whereas the introduction of 100 μg of MAN, even on three occasions, did not. To explore the dose-response and time of administration of MAN further, a second experiment was performed in which 1,000, 500, and 250 μg of MAN were administered twice, 1 week before and on the day of the initiation of immunization, or once only, either 1 week before or on the day of the initiation of immunization. The data are summarized in the lower portion of Fig. 1. Clearly, all doses of MAN induced significant levels of suppression, whether administered once or twice prior to cutaneous immunization. The least suppressive dose was 250 μg administered once on the day of the initiation of immunization. In subsequent experiments, 500 μg administered on one occasion was selected as the optimal dose, and MAN was administered 1 day prior to the first or second

TABLE 2. Comparison of *C. albicans* GP, MAN, BEX, and SCS as antigens in an in vitro lymphoproliferative assay using cells from lymph nodes draining the site of the cutaneous lesion in colonized, cutaneously inoculated CBA/J male or female mice or from spleens of similarly treated mice

Expt[a]	IG[b]	CUT[c]	Site[d]	Mean total cpm± SEM of [^{3}H]thymidine incorporated with:						
				CONT[e]	GP	MAN	BEX	SCS	CONT	Mitogen[f]
I	+	+	LN	623 ± 112	12,121 ± 342	3,770 ± 96	9,120 ± 133	10,507 ± 487	667 ± 127	34,660 ± 413
			S	1,407	6,301	3,437	7,455	6,085	794	15,660
	+	−	LN	540 ± 81	484 ± 37	829 ± 59	1,102 ± 38	630 ± 75	210 ± 12	32,183 ± 1,830
			S	828	681	267	1,093	1,158	697	17,302
	−	+	LN	532 ± 139	693 ± 62	675 ± 102	554 ± 38	612 ± 86	363 ± 47	19,110 ± 1,682
			S	618	630	409	1,428	2,083	676	12,167
	−	−	LN	330 ± 33	392 ± 28	361 ± 22	407 ± 117	507 ± 30	263 ± 74	25,607 ± 1,816
			S	507	367	308	648	586	534	18,232
II	+	+	LN	126 ± 9	2,959 ± 467	2,486 ± 455	ND[g]	5,336 ± 1,399	1,022 ± 144	43,816 ± 1,044
			S	1,132 ± 211	4,624 ± 733	3,710 ± 257	ND	5,237 ± 593	2,226 ± 599	28,266 ± 1,267
	+	−	LN	172 ± 15	381 ± 79	195 ± 19	ND	180 ± 13	331 ± 12	31,419 ± 2,889
			S	754 ± 131	648 ± 111	642 ± 169	ND	1,010 ± 256	726 ± 27	23,309 ± 1,533
	−	+	LN	355[h]	15,259	3,154 ± 941	ND	6,749 ± 505	764 ± 111	47,190 ± 2,255
			S	465 ± 67	811 ± 86	977 ± 84	ND	1,683 ± 119	744 ± 13	25,233 ± 547
	−	−	LN	241[h]	498 ± 40	464 ± 9	ND	787 ± 128	479[h]	34,826[h]
			S	1,106 ± 188	ND	875 ± 127	ND	1,094 ± 201	483[h]	31,725 ± 1,122
III	+	+	LN	853 ± 61	5,440 ± 392	1,793 ± 266	4,617 ± 17	6,165 ± 490	622 ± 54	33,045 ± 2,090
			S	1,125	3,382	2,633	4,054	2,573	3,413	9,916
	+	−	LN	354 ± 53	885 ± 134	525 ± 52	1,038 ± 126	1,051 ± 137	289 ± 30	17,544 ± 95
			S	916	5,632	2,100	5,109	5,067	1,340	20,933
	−	+	LN	330 ± 61	1,130 ± 248	894 ± 258	1,860 ± 252	792 ± 108	238 ± 33	17,544 ± 95
			S	547	1,160	468	1,354	1,701	824	16,348
	−	−	LN	360 ± 29	560 ± 26	663 ± 47	584 ± 58	640 ± 3	156 ± 17	16,746 ± 656
			S	762	814	253	183	649	832	25,447

[a] The assay was performed 6 or 12 days following the cutaneous inoculation of female mice (experiments I and III) or 9 days following the cutaneous inoculation of male mice (experiment II).
[b] IG, Intragastric inoculation of viable *C. albicans* at 6 days of age.
[c] CUT, Cutaneous inoculation of viable *C. albicans* at 7 to 10 weeks of age.
[d] LN, Lymph nodes; S, spleen.
[e] CONT, Medium alone.
[f] Cells were stimulated with phytohemagglutinin at 6 and 12 days and with ConA at 9 days.
[g] ND, Not done.
[h] Averages of duplicate wells only; all other data are means based on triplicate wells.

cutaneous inoculation with *C. albicans*. In early experiments (data not shown), groups of unsensitized mice receiving MAN i.v. were tested for the potential development of DH in response to MAN. None of the animals inoculated with MAN developed DH responses; therefore, that group of animals was deleted from subsequent experiments.

The next series of experiments was designed to determine if MAN administered during the course of immunization, i.e., after the priming phase but prior to the booster inoculation, would suppress the subsequent development of DH, as was observed when it was administered prior to the initiation of immunization. A representative experiment is presented in Fig. 2. Included in this experiment as a positive control for suppression were two groups of animals immunized by the protocol involving two cutaneous inoculations, one of which was treated with MAN prior to the initiation of immunization. The second portion of the experiment involved i.v. treatment with MAN 1 day prior to a single cutaneous inoculation with viable *C. albicans* of uncolonized CBA/J male mice and sex- and age-matched mice colonized with *C. albicans*. Footpad testing was done 1 week following the cutaneous inoculation. We have shown previously that maximum DH responses were induced in colonized mice by this protocol (10). The administration of MAN prior to the cutaneous booster inoculation reduced the DH response to that observed in uncolonized animals receiving only a single cutaneous inoculation with *C. albicans*. The latter animals also developed significantly reduced responses if treated with MAN prior to the cutaneous challenge. DH responses in the uncolonized animals treated with MAN prior to the first of two cutaneous inoculations, i.e., the positive-control animals, were significantly suppressed as expected. Therefore, MAN affected memory cells as well as cells undergoing a primary response to antigen.

Specificity of suppression induced by MAN. A final series of experiments was undertaken to determine whether the suppression induced by MAN was MAN specific or if responses to other candidal antigens were influenced as well. Two representative experiments are presented in Fig. 3. The first experiment involved adult CBA/J female mice treated with 500 μg of MAN i.v. 1 day prior to the first or second cutaneous inoculation with *C. albicans*, and the second experiment involved the treatment of adult CBA/J male mice with 1,000 μg of MAN i.v. 1 day prior to the first of two cutaneous inoculations with *C. albicans*. In the first experiment MAN and GP were compared as antigens, whereas in the second experiment, MAN and BEX were compared. Administration of MAN before or during the immunization process significantly suppressed DH responses to itself as well as to GP, a glycoprotein containing MAN (32), but responses to BEX, a membrane antigen devoid of MAN, were not significantly suppressed at the 24-h observation point. There was a significant difference between the MAN-

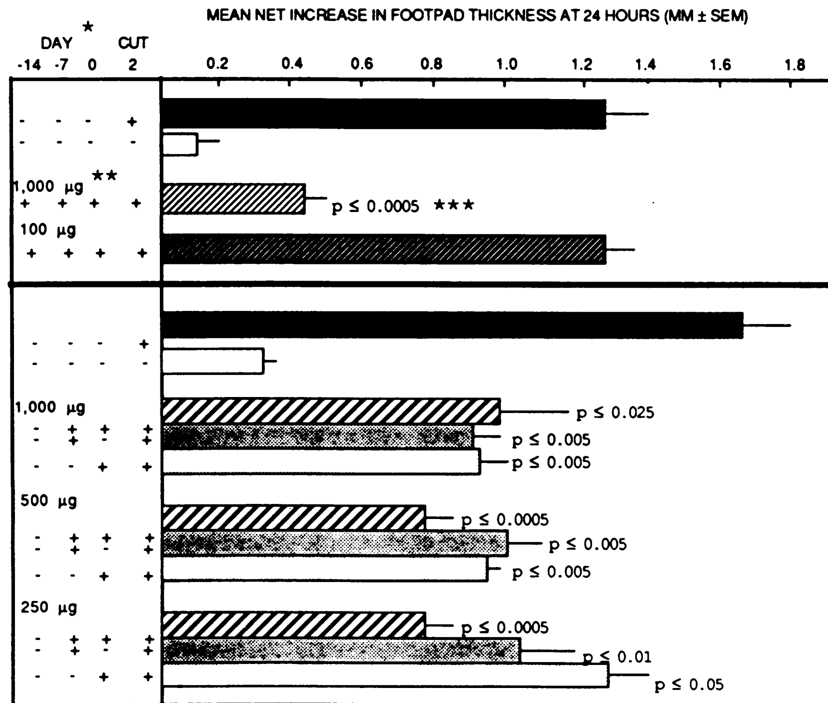

FIG. 1. Twenty-four-hour footpad responses to 30 µg of MAN in CBA/J adult male mice immunized by the inoculation twice cutaneously (CUT) of *C. albicans* and treated i.v. with varying doses of MAN on selected days prior to the initiation of immunization. Symbols: ★, day with respect to the first of two cutaneous inoculations of viable *C. albicans*; ★★, daily dose of MAN administered i.v.; ★★★, statistical significance based on Student's *t* test (n = 4 to 6). SEM, Standard error of the mean.

treated and untreated groups tested with BEX at 48 h, however.

Because of the 48-h observation noted above for MAN-treated, BEX-tested animals, two additional experiments were performed, one in adult CBA/J males and the other in adult CBA/J females, each inoculated i.v. with 500 µg of MAN 1 day prior to the first of two cutaneous inoculations with *C. albicans*. MAN-treated, MAN-tested mice had significantly suppressed DH responses in both experiments, and no suppression was observed in the MAN-treated, BEX-tested male mice, but there was some suppression noted in the female mice. The level of suppression noted in the female MAN-treated, BEX-tested mice, however, was less than that observed with the female MAN-treated, MAN-tested animals. The suppression observed in response to testing with BEX may result from some contamination of the BEX preparation with MAN, although we have no evidence to support this hypothesis at this time. To investigate this phenomenon further, the reverse experiment, i.e., treatment of mice i.v. with BEX prior to the initiation of sensitization, followed by footpad testing with BEX and MAN, was performed. Suppression was noted to BEX in BEX-treated mice, but there was no suppression to GP in similarly treated mice. For example, the 24-h response to GP in untreated immunized animals was 1.15 ± 0.11 mm, whereas that in BEX-treated immunized mice was 1.14 ± 0.09 mm.

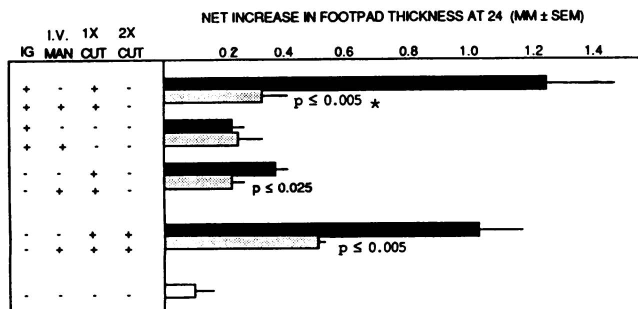

FIG. 2. Twenty-four-hour footpad responses to MAN in CBA/J adult male mice immunized by the inoculation twice cutaneously (CUT) of *C. albicans* or in CBA/J male mice which were colonized as infants (IG [intragastric]) and inoculated once cutaneously as adults. MAN was administered 1 day prior to the first of one or two cutaneous inoculations. Symbol: ★, statistical significance based on Student's *t* test (n = 4 to 6). SEM, Standard error of the mean.

DISCUSSION

The data presented here demonstrate that MAN is a suitable antigen for the detection of CMI in vivo, and they confirm in an animal model that MAN introduced to the lymphoid tissue via the circulation can suppress the development of CMI responses detectable in vivo by challenge with the same antigen or another antigen containing MAN as a major component. As has been suggested by previously published data with human cells (2, 19), MAN was determined to be a poor antigen in vitro for the detection of CMI responses. This latter phenomenon may relate to the fact that in vitro, suppressive and stimulatory responses may be competing against each other (38). It is not clear at this time which component of the MAN extract, i.e., MAN, protein, or the intact mannoprotein, was responsible for the detection, as well as the suppression, of DH. Resolution of that issue awaits the complete separation of the MAN component from the protein component. Carrow and Domer (6), however, demonstrated that treatment with papain or heat abro-

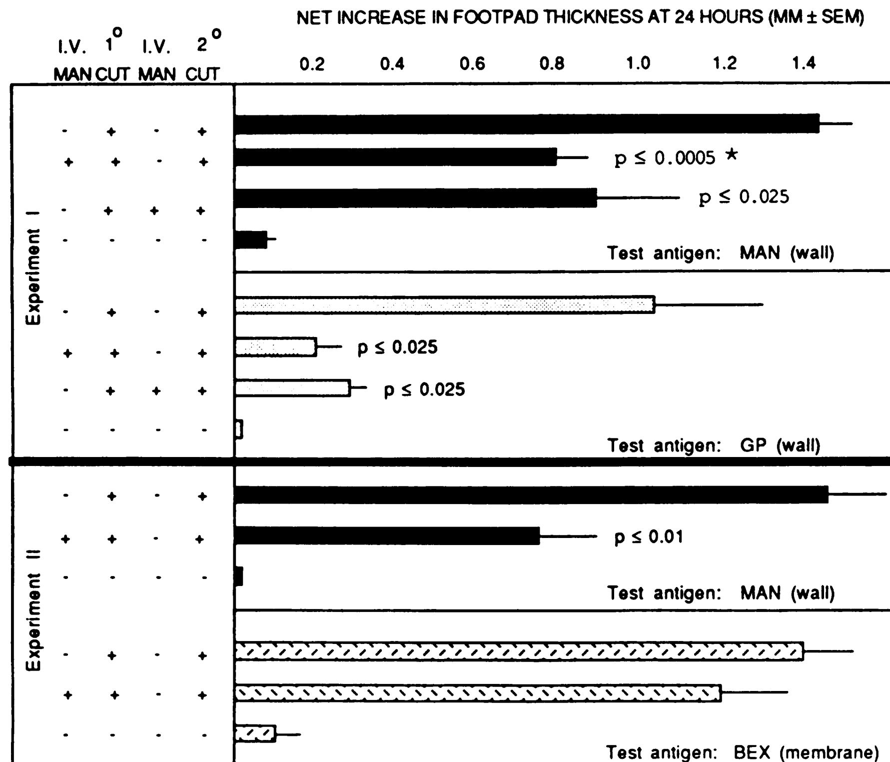

FIG. 3. Twenty-four-hour footpad responses to MAN and GP in CBA/J adult male mice (experiment I) and to MAN and BEX in CBA/J adult female mice (experiment II). MAN was administered prior to the first or second of the two cutaneous (CUT) inoculations with *C. albicans*. Symbol: ★, statistical significance based on Student's *t* test. SEM, Standard error of the mean.

gates the ability of GP, a MAN-containing glycoprotein (32) from *C. albicans* cell walls, to induce suppression. Moreover, Domer and Moser (11) had shown previously that the protein component of GP is essential to the elicitation of DH, in that proteolytic treatment of GP completely abrogates its ability to function as an antigen. Periodate treatment, however, reduces the normal DH response as well, suggesting that the carbohydrate moiety may contribute to the specificity.

The clinical correlate of these studies has been the demonstration that many patients with serious forms of candidiasis, in particular CMCC, have depressed CMI associated with circulating inhibitors, as judged by both in vivo testing for DH and in vitro testing by lymphocyte stimulation (LS) (1, 5, 16, 18, 28, 39, 47). Perhaps the most definitive clinical study linking circulating MAN to in vivo immunosuppression was that of Fischer et al. (16). A serum inhibitor was detected in 6 of 23 patients with CMCC, and a MAN-containing polysaccharide was isolated from the serum of one of the patients which suppressed the response of peripheral blood mononuclear cells derived from normal subjects. Moreover, in studies published later, Fischer et al. (17) presented data implicating the monocyte in the suppressive phenomenon observed initially in the CMCC patients. Monocytes from two of the patients were defective in processing MAN, but uptake of MAN into the monocytes was not altered nor were uptake and processing of an unrelated antigen, pneumococcal polysaccharide type III.

The cellular basis of the mechanism of suppression observed here in vivo is not clear at this time, although studies are currently in progress to attempt to resolve the issue. As indicated above, Fischer et al. (17) felt that the suppression observed in their patients with CMCC was related to defective monocyte handling of MAN, and they were unable to obtain evidence for the involvement of a suppressor cell in the phenomenon. Others (3, 42, 50), however, presented evidence for the involvement of suppressor T lymphocytes in vitro by incubating normal human peripheral blood lymphocytes with a crude *Candida* commercial antigen, a complex mixture known to contain MAN (34), or a laboratory-produced candidal antigen designated MPPS containing mannose, glucose, and protein (41). The suppressor cells induced were identified on the basis of the ability to inhibit the response of normal lymphocytes to *Candida* antigen. The two phenomena, i.e., monocyte and T-cell suppression, need not be mutually exclusive, in that the macrophage may, in fact, initiate a cascade of events that leads to the production of T-suppressor cells (8, 13, 14, 29, 31). Alternatively, MAN persisting in vivo as a consequence of poor degradation may activate T-suppressor cells directly. Although the data presented by us here involve CMI responses exclusively, Durandy et al. (14) showed that T cells taken from CMCC patients in the midst of active disease suppress in vitro antibody formation by peripheral blood lymphocytes from healthy subjects. Monocytes are required for T-suppressor cell activation, and both $CD8^+$ and $CD8^-$ T lymphocytes are involved.

Other investigators have described the production of T-suppressor lymphocytes in response to circulating fungal antigen. For example, Murphy and co-workers (35–37) described a T-suppressor cell circuit which was responsible for suppression of induction and elicitation of DH responses when cryptococcal antigen was administered i.v., and Jimenez-Finkel and Murphy (22, 23) described the induction

of such cells in response to soluble *Paracoccidioides brasiliensis* antigen. Although the specific population of cells has not yet been defined, suppression of T lymphocyte responses to *Coccidioides immitis* antigen administered i.v. can be transferred with splenocytes from animals treated with soluble *Coccidioides* antigen (9).

Despite the fact that the mechanism of suppression described here has not been determined, the fact that MAN exerted its effects when administered 1 week before sensitization was begun suggests that it has a long-lasting effect on a select population of cells. We hypothesize, although we have no data to support the hypothesis at the moment, that MAN is cleared relatively rapidly from the circulatory system of the mouse, so that the MAN must interact with the responsible cell(s) within a relatively short time after i.v. introduction. Jones (24) observed rapid clearance of a large quantity in a relatively short interval in rabbits, i.e., 5 mg is cleared in less than 48 h. While the 250 to 500 μg used in our studies seems high in proportion to the size of a mouse, MAN administered i.v. via the tail vein may become complexed with MAN-binding proteins (4) or be removed from the circulation via other mechanisms as it traverses the circulatory system, leaving relatively small amounts for interaction with the lymphoid cells which suppress subsequent responses. During serious disease in humans, MAN would probably be sloughed regularly from foci containing large numbers of organisms, continually seeding the circulatory system, thus continuously activating suppressor phenomena. Alternatively, high concentrations of MAN may occur in or near foci of infection during disseminated disease, and if such foci are in the vicinity of the appropriate lymphoid cells, suppressor phenomena could be generated in this manner.

Finally, since it is known that MAN preparations contain distinct fractions which differ with respect to size, charge, and ability to modulate non-*Candida* immune responses (12), future studies need to be focused on that component(s) responsible for initiating and maintaining the suppression, as well as on the lymphoid cell(s) responsible for the observed modulatory effect.

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