ACQUIRED IMMUNITY TO CANDIDIASIS IN MICE[1]

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ABSTRACT

HASENCLEVER, H. F. (National Institute of Allergy and Infectious Diseases, Bethesda, Md.) AND WILLIAM O. MITCHELL. Acquired immunity to candidiasis in mice. J. Bacteriol. **86**:401–406. 1963.—Some protection to chronic candidiasis in mice was produced by sublethal intraperitoneal infection with *Candida albicans* and by the injection in incomplete Freund's adjuvant of nonviable *Coccidioides immitis* spherule fragments. Other pathogenic or nonpathogenic fungi produced little or no protection. *Salmonella enteritidis* lipopolysaccharide, known to protect mice against the acute toxic manifestations of *C. albicans*, had no effect upon chronic candidiasis. A factor active in vitro against the growth of *C. albicans* was shown to be in the serum of resistant mice.

A number of studies have been published pertaining to acquired immunity to infection with *Candida albicans*. Hurd and Drake (1953) reported that rabbits could not be actively or passively immunized against fatal infection with this yeast, and Winner (1956) reported similar results. Fomina and Stephanishcheva (1958) reported both active and passive protection in mice against subacute but lethal *C. albicans* infection, and Mourad and Friedman (1961) also presented evidence of active protection in mice.

Studying acquired resistance or tolerance to the toxic manifestations of *C. albicans*, Hasenclever and Mitchell (1962*a*, *b*) found that little or no antigenic specificity was associated with protection. The investigation reported here was undertaken to determine whether resistance to subacute or chronic candidiasis, where the challenge dose was 100 times less than that used for acute toxicity studies, was also nonspecific in nature.

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MATERIALS AND METHODS

The animals used in these studies were female general-purpose Swiss white mice obtained from the Animal Production Section of the National Institutes of Health. They weighed 15 to 19 g when the experiments were initiated.

The strain of *C. albicans* used was B311, antigenic group A. It was isolated from a fatal case of candidiasis, has been maintained on artificial culture media for 4.5 years, but still possesses a high level of mouse virulence.

C. albicans yeast cells for intravenous challenge were obtained from 2% glucose, 1% neopeptone stationary broth cultures incubated at 30 C for 2 to 3 days. Plate counts were used to determine the size of the challenge dose. Yeast cells for immunization were harvested from 2% glucose, 1% neopeptone agar slants incubated at 30 C for 1 to 2 days. *Aspergillus niger*, *Fusarium oxysporum*, and *Sphaerostilbes repens* were cultured at 30 C in glucose-neopeptone broth for 7 days. The mycelial growth was removed, treated in a Waring Blendor for 2 to 3 min, washed with 0.85% NaCl solution, and used to determine whether any protection in mice could be elicited by nonpathogenic fungi.

Yeastlike cells of *Blastomyces dermatitidis* and *Histoplasma capsulatum* were obtained from cultures grown on blood-glucose-cysteine-agar slants incubated at 37 C for 4 to 5 days. *Coccidioides immitis* spherules, obtained from George Lones, were cultured in Converse medium. Where nonviable preparations were used, with the exception of *C. immitis*, viability was destroyed by heating at 65 C for 4 to 6 hr. *C. immitis* spherules were treated with 1% formalin solution for 24 hr at 30 C. They were then washed, passed through a French pressure cell at 25,000 psi, centrifuged, and the fragments retained and used for injection. Whole cells of the yeastlike fungi were utilized for this purpose.

Where nonviable fungal cells or preparations were used, 1 mg (dry weight) of the material sus-

pended in 0.1 ml of incomplete Freund's adjuvant was given subcutaneously per mouse. The injections were given 14 and 7 days before challenge. Mice in X-ray and thorium dioxide experiments were injected at 20 and 13 days, and some groups were X-rayed 6 days before challenge. Viable yeast cell suspensions were made up in saline, and from 10^7 to 2×10^7 cells were given intraperitoneally 14 and 7 days before challenge. The same weight of powdered Pyrex or alumina in saline was injected as for the nonviable fungal preparations, and the same time schedule was employed.

The *Salmonella enteritidis* lipopolysaccharide and incomplete Freund's adjuvant were purchased from Difco. The endotoxin was dissolved in sterile distilled water, and 30 μg were injected intraperitoneally at the times indicated in Table 5.

The in vivo growth rates of *C. albicans* in mice injected with *C. immitis* spherule fragments and in control mice were determined. The animals were challenged intravenously with approximately the same number of *C. albicans* cells as were the mice for other immunity experiments. Three mice from each group were killed, and the kidneys were quantitatively cultured at the various time intervals indicated in Fig. 1. All animals still alive after 28 days were killed, and their kidneys were cultured.

In addition, the kidneys of all mice that died during the experiment were plated; 2% glucose-Trypticase Soy Agar (BBL) was the medium used for the plating procedures.

For the X-ray studies, a dual-tube Westinghouse machine operating at 200 kv and 15 mamp, with 0.25-mm Cu and 0.55-mm Al filters, was used. The mice were placed 54 cm from the X-ray tubes and were exposed to 118.2 r for 3.6 min for a total body irradiation of 425 r. They were challenged 6 days later.

The effect of reticuloendothelial blockade in control mice and mice treated with thorium dioxide (Thorotrast; Testagar & Co., Inc., Detroit, Mich.) was studied. This colloidal preparation contained 24 to 26% thorium dioxide by volume, and 0.2 ml was injected intravenously 1 to 2 hr before challenge.

The in vitro inhibitory activity of sera from *C. albicans*-infected or *C. immitis*-injected mice was determined by use of 10% serum in 0.25% Trypticase Soy Broth. Sera were collected from mice that had been injected in a manner identical to those in other groups used for immunity studies. The sera from control or treated mice were diluted in the Trypticase Soy Broth, and 50 μg of chloramphenicol per ml of broth-serum mixture were added. An inoculum of approximately 100 yeast cells per ml of serum medium was used. The tubes containing the serum broth were incubated at 37 C, and pour plates in triplicate were made from appropriate dilutions of samples from each tube after inoculation, at the times indicated in Fig. 3.

RESULTS

The data shown in Table 1 indicate the amount of protection against chronic *C. albicans* infections conferred by the intraperitoneal injection of a number of different yeasts. Mice preinfected with the homologous *C. albicans* strain apparently developed the highest level of resistance, those with *Candida stellatoidea* and *Candida tropicalis* intermediate levels, and the mice that received *Candida parapsilosis*, *Torulopsis glabrata*, and *Saccharomyces cerevisiae* demonstrated little if any protection.

Table 2 shows the effect on chronic candidiasis of *C. immitis* spherule fragments suspended in incomplete adjuvant or saline and of nonviable *C. albicans* cells in incomplete adjuvant or saline. Mice preinfected intraperitoneally with *C. albicans* were included as positive controls. These results illustrate the ineffectiveness of nonviable *C. albicans* cells in producing any acquired resistance. The injection of *C. immitis* spherule fragments in incomplete adjuvant protected mice, as did intraperitoneal preinfection with *C. albicans*.

The results in Table 3 indicate the protection conferred by the injection in mice of several nonviable antigenically unrelated pathogenic or non-

TABLE 1. *Effect of prior injection with viable antigenically related yeasts on chronic candidiasis in mice produced by intravenous injection of 8×10^4 viable units of Candida albicans*

Yeast injected	No. dead/total no.	Per cent mortality
C. tropicalis	19/30	63
C. stellatoidea	14/29	48
C. parapsilosis	25/30	83
C. albicans	10/29	34
Torulopsis glabrata	24/30	80
Saccharomyces cerevisiae	24/30	80
Controls	28/29	97

pathogenic fungi. Nonviable *C. albicans* cells are also included. In this experiment, as in the one in Table 2, *C. immitis* gave comparatively good protection. Mice that received *H. capsulatum* developed some resistance to chronic candidiasis, whereas the other fungi tested stimulated little or no definite protection.

Table 4 indicates the effect upon chronic infection with *C. albicans* of injection in mice of inert substances. Neither ground Pyrex nor alumina caused any definite enhancement of resistance to *C. albicans*; however, the level of protection produced by preinfection with the homologous strain or by injection of *C. immitis* spherule fragments was less than in other experiments. This illustrates that there is some variability in the level of protection developed in mice after injection of these agents.

In earlier studies, Hasenclever and Mitchell (1962*b*, 1963) reported that *S. enteritidis* or *Salmonella typhosa* lipopolysaccharides protected mice against the acute toxicity of *C. albicans*. Consequently, experiments were done (Table 5) to determine whether these endotoxins would stimulate resistance in mice to chronic candidiasis. None of the schedules of injections that produced resistance in mice to *C. albicans* toxicity produced resistance to chronic disease. In other experiments not shown here, larger doses of endotoxin were likewise ineffective.

The results in Table 6 show the effect of X ray and thorium dioxide in *C. immitis*-injected mice

TABLE 2. *Effect of prior injection with Coccidioides immitis spherule fragments and viable or nonviable Candida albicans yeast cells on chronic candidiasis in mice produced by intravenous injection of 1.5 × 10⁵ viable units of C. albicans*

Injected with	No. dead/ total no.	Per cent mortality
C. immitis spherule fragments in 0.85% NaCl	21/30	70
C. immitis spherule fragments in incomplete adjuvant	7/30	23
Viable *C. albicans*	5/27	19
Nonviable *C. albicans* in 0.85% NaCl	23/29	80
Nonviable *C. albicans* in incomplete adjuvant	28/30	93
Incomplete adjuvant	25/29	86

TABLE 3. *Effect of prior injection with nonviable pathogenic or nonpathogenic fungi on chronic candidiasis in mice produced by intravenous injection of 1.5 × 10⁵ viable units of Candida albicans*

Fungus injected	No. dead/ total no.	Per cent mortality
Aspergillus niger	17/29	59
Fusarium oxysporum	23/32	72
Sphaerostilbes repens	18/30	60
Histoplasma capsulatum	12/29	41
Cryptococcus neoformans	23/29	79
Blastomyces dermatitidis	16/30	53
Candida albicans	19/30	63
Coccidioides immitis	8/29	27
Incomplete adjuvant	26/29	90
Controls (0.85% NaCl)	30/30	100

TABLE 4. *Effect of prior injection of inert substances on chronic candidiasis in mice produced by intravenous injection of 1.3 × 10⁵ viable units of Candida albicans*

Injected with	No. dead/ total no.	Per cent mortality
Powdered Pyrex	22/29	75
Alumina	26/29	90
Coccidioides immitis	14/30	47
Candida albicans	11/27	41
Controls (0.85% NaCl)	28/30	93

TABLE 5. *Effect of prior injection of Salmonella lipopolysaccharide on chronic candidiasis in mice produced by intravenous injection of Candida albicans*

Lipopolysaccharide schedule prior to challenge		Mortality rate with challenge dose of			
		1.5 × 10⁵		1.5 × 10⁴	
No. of doses	Administered at	No. dead/ total no.	Per cent mortality	No. dead/ total no.	Per cent mortality
	days				
3	7, 3, 1	27/30	90	12/29	41
1	7	27/28	96	—*	—*
1	1	25/30	83	13/30	43
3†	7, 3, 1	25/29	86	14/30	47

* This group of mice was inadvertently destroyed.

† This control group was given 0.1-ml doses of water.

TABLE 6. *Effect of X ray or thorium dioxide on resistance to chronic candidiasis produced by intravenous injection of 6 × 10^4 viable units of Candida albicans*

Mice treated with	No. dead/ total no.	Per cent mortality
Coccidioides immitis, thorium dioxide	19/30	63
C. immitis, X ray	29/29	100
C. immitis	9/30	30
Incomplete adjuvant, thorium dioxide	28/30	93
Incomplete adjuvant, X ray	30/30	100
Incomplete adjuvant	23/30	77
Untreated controls	26/30	87

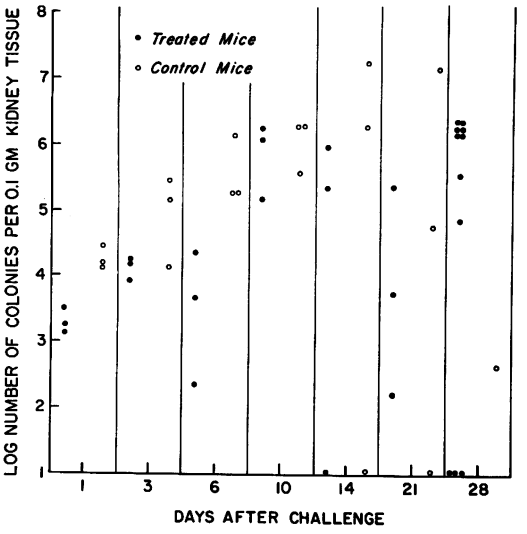

FIG. 1. *In vivo Candida albicans population in kidneys of sacrificed Coccidioides immitis-injected and control mice. Each dot or circle represents the number of viable yeast cells or aggregates cultured from a single animal.*

challenged with approximately 6 × 10^4 *C. albicans* cells. This method of producing resistance was chosen because a nonviable, but effective, preparation was employed. X ray completely destroyed all acquired protection. Although the effect of thorium dioxide was transient, injection of this substance reduced the level of acquired resistance. These results indicate the probable importance of cellular activity in this state of resistance. We cannot eliminate the possibility that these two methods, believed to inhibit function of the reticuloendothelial system, have a broader physiological effect that contributes to the loss of acquired resistance in some other manner.

Results of the preceding experiments indicated that injection of *C. immitis* spherule fragments in incomplete adjuvant or the intraperitoneal injection of viable *C. albicans* cells produced the greatest amount of acquired resistance to chronic candidiasis. This resistance was observed in the form of a greater survival rate in treated mice. Another way to measure enhanced protection is to follow the growth rate of the challenge yeast in the tissues of treated and control mice. Mice injected with the *C. immitis* preparation and the appropriate controls were utilized for this purpose. The in vivo multiplication of *C. albicans* in the kidneys of treated and untreated sacrificed mice is shown in Fig. 1. Since most of the multiplication of *C. albicans* occurs in the kidney, this was the only tissue plated. The values obtained from the treated mice were generally below those from the control group. Figure 2 shows the number of *C. albicans* viable units in the kidneys of mice that died of overwhelming disease after challenge. Again, the number of viable units in the treated mice as a whole was slightly below that for the control group.

In vitro growth studies with *C. albicans* in sera from mice injected with C. *immitis* or viable *C. albicans* indicated the presence of a serum factor that might have been partially responsible for the state of acquired resistance. Sera for this experiment were obtained from mice injected with the

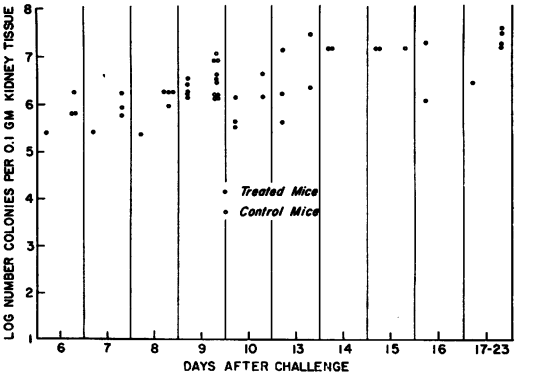

FIG. 2. *In vivo Candida albicans population in kidneys of Coccidioides immitis-injected and control mice that died during the experiment. Each dot or circle represents the number of viable yeast cells or aggregates cultured from a single animal.*

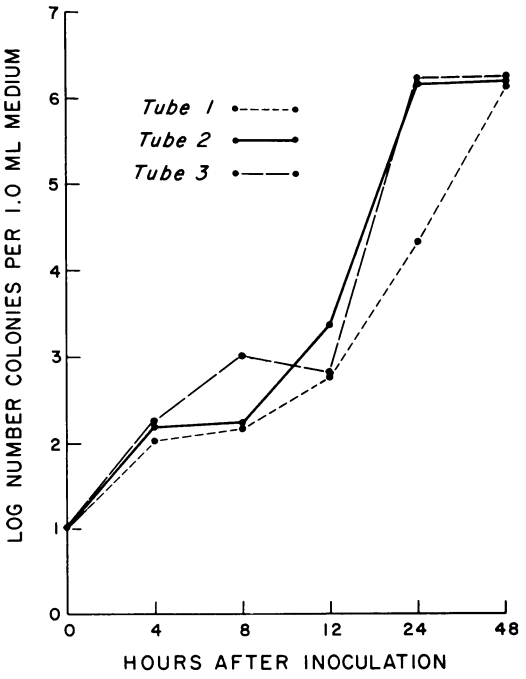

FIG. 3. *Effect of serum from Coccidioides immitis-injected mice on the growth of Candida albicans in vitro. Tube 1, serum from C. immitis-injected mice; tube 2, serum from control mice; tube 3, no serum. Each point on the graph represents the average of three pour plates per dilution of medium. Sera from C. albicans-infected mice gave similar results.*

C. immitis preparation. The same schedule of injection was used as for earlier studies. Sera from saline-injected mice were used as controls. The effect of these sera upon the growth rate of *C. albicans* is shown in Fig. 3. At 24 hr after inoculation, sera from treated mice had suppressed growth 100-fold when compared with sera from control mice and medium controls without serum. At 48 hr, however, the serum activity was no longer apparent. Sera from *C. albicans*-injected mice exerted a similar in vitro effect.

DISCUSSION

The highest level of resistance in mice to chronic candidiasis can be produced by preinfection with *C. albicans* or by the injection of nonviable *C. immitis* spherule fragments in incomplete adjuvant. Preinfection with *C. stellatoidea* or *C. tropicalis* and the injection of nonviable *H. capsulatum* in incomplete adjuvant elicited lower levels of protection. The other pathogenic and nonpathogenic fungi and the inert irritants tested produced little or no protection.

The studies with endotoxin indicated that, as given, this substance did not stimulate the development of acquired resistance in mice against chronic but lethal candidiasis. It is possible, however, that the protection produced is too transient to be effective. Our earlier studies (Hasenclever and Mitchell, 1962*b*), with lipopolysaccharide-induced protection against acute toxicity, indicated that the tolerant effect was no longer demonstrable 10 to 14 days after injection. This substance cannot be given to mice after challenge with *C. albicans*, owing to infection hypersusceptibility (Hasenclever and Mitchell, 1963). Box and Briggs (1961) reported increased hypersusceptibility to endotoxin in mice infected with *H. capsulatum*, and Suter (1962) extensively studied hyperactivity to endotoxin in *Mycobacterium tuberculosis*-infected mice.

Our earlier results (Hasenclever and Mitchell, 1962*b*), which indicated that the duration of protection induced by endotoxin to *C. albicans* toxicity was less than 14 days, and these results showing that endotoxin produced no protection against chronic candidiasis are different from those reported by other investigators working with other infectious agents. Dubos and Schaedler (1956) reported long-lasting protection conferred to mice against chronic staphylococcal or *M. tuberculosis* infection. Louria (1960) showed resistance in mice to cryptococcosis after endotoxin injection. There appears to be some difference between acquired resistance in mice to acute lethal toxicity and to chronic lethal infection due to *C. albicans*. Lipopolysaccharide confers protection from toxicity only, whereas the injection of *C. immitis* spherule fragments or viable *C. albicans* stimulates protection from both acute toxic or chronic disease. Although the injection of mice with *C. immitis* spherules produces protection from *C. albicans* toxicity, it does not produce tolerance or protection in mice against lethal amounts of *S. enteritidis* endotoxin. Mice infected with *C. albicans* are more susceptible to endotoxin than are normal mice.

The studies of the in vivo growth rates of *C. albicans* in *C. immitis*-injected and control mice (Fig. 1 and 2) indicate only slight differences and do not permit clear concise conclusions. However, when the mortality results in the various experiments are examined (i.e., Table 2: *C. immitis*-

injected, 27%, controls, 90%; Table 4: *C. immitis*-injected, 47%, controls, 93%; Table 6: *C. immitis*-injected, 30%, controls, 87%), this possibly unimpressive difference appears to take on more significance.

The presence of *C. albicans* growth-inhibiting substances in the sera of *C. immitis*-injected mice may also contribute to the state of acquired resistance. Other studies with sera obtained from mice injected with endotoxin indicated the presence of similar inhibitory factors (Hasenclever and Mitchell, 1963). Whitby et al. (1961) reported the formation, in the sera of mice, of specific-lytic antibodies to gram-negative bacteria after the injection of endotoxin. There was some heterologous response, but the specific response was stronger. Although our work (Hasenclever and Mitchell, 1963) showed that *S. enteritidis* endotoxin stimulated mice to produce serum-inhibitory substances for *C. albicans*, antigenic similarity between these two microorganisms is not known to exist. More investigation is necessary to elucidate the nature of these inhibitory factors found in the sera of mice after injection of endotoxin, *C. immitis* spherule fragments, or viable *C. albicans*.

These studies indicate that acquired protection in mice against chronic candidiasis can be produced. The degree of resistance observed is not of a high level of magnitude and is somewhat variable. Studies at the cellular level and on serum factors may be helpful in explaining the nature of this resistance.

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