ATTEMPTS TO IMMUNIZE MICE AGAINST SPOROTRICHOSIS*

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Investigation of altered resistance of laboratory animals to the systemic mycoses has centered largely on coccidioidomycosis (1, 2), and histoplasmosis (3, 4). The lack of a dependable nontoxic chemotherapeutic agent, and the high incidence of these two diseases have undoubtedly influenced research on them. Sporotrichosis, on the other hand, can be treated effectively with potassium iodide, although treatment must be extended over several months to insure cure. The incidence of this infection is not as high as that of coccidioidomycosis or histoplasmosis, but under some circumstances it has caused very large epidemics (5).

The early studies of Jessner (6) with sporotrichosis in rats indicated that a partial immunity existed to cutaneous reinfection. Kesten and Martenstein (7) reported that, after intracardial injection of *Sporotrichum schenckii* spores, blood cultures were sterile earlier in animals with previous experimental cutaneous sporotrichosis than in animals without previous exposure to the fungus.

This paper reports results obtained from attempts to increase the resistance of Swiss white mice to sporotrichosis.

MATERIALS AND METHODS

Strain and Cultural Procedures

The strain of *S. schenckii* used throughout this study was isolated from a case of human sporotrichosis. Yeast-like growth of this organism from blood glucose cysteine agar, incubated 4 days at 37° C. was used for the preparation of the various vaccines, or for challenge. Conidiospore suspensions for rabbit immunization were prepared from *S. schenckii* cultures grown on modified† Sabouraud's agar slants incubated at 30° C. for 7 days.

Cultures from tissues were made by removing the spleen and a portion of the liver aseptically from chloroformed animals, macerating them on Sabouraud's agar slants, and incubating for two weeks at 30° C. Four slants were inoculated per animal, i.e., 2 slants from spleen and 2 from liver.

Vaccines

The vaccine preparations were of 4 general categories: 1. nonviable suspensions of *S. schenckii* yeast-like cells; 2. an acetone extract of the yeast-like cells; 3. saline extracts or homogenates from *S. schenckii* infected mouse spleens; and 4. viable suspensions of *S. schenckii*.

Three nonviable vaccines were prepared as follows: 1. Yeast cells were suspended in a 2% formalin solution at 37° C. for 48 hours and subsequently washed several times with sterile physiological saline; 2. *S. schenckii* cells were left overnight in acetone at 4° C., then washed with saline; and 3. yeast-like cells were suspended at 55° C. for 40 minutes and washed. These vaccines were proved to be sterile by culturing, and were used after appropriate dilution.

The tissue extracts and homogenates were prepared from the spleens of mice infected intravenously with *S. schenckii* by grinding in a sterile mortar at a spleen-saline ratio of 1:4 weight/volume. Pour plates made in triplicate with tenfold dilutions of infected splenic tissue indicated that there were approximately 13.3×10^6 viable *S. schenckii* cells per 0.1 gram. The homogenate was heated for an hour at 55° C.; the coarse tissue particles were allowed to settle for 15 minutes, and the supernatant was removed and tested as an immunizing agent. The extract was obtained by the centrifugation of unheated ground spleen-saline mixture, followed by Seitz filtration as a means of sterilization. Control extracts and homogenates were made from spleens of normal mice following the same procedure. Cultures from these preparations produced no growth when tested for sterility.

The acetone extract of yeast-like cells of *S. schenckii* was produced by treating the 4-day growth from 6 blood glucose cystine agar slants with 20 ml. of acetone overnight at 4° C. The acetone was decanted from the yeast-like cells and evaporated on an electric plate set at low temperature. The material remaining after evaporation was reconstituted with 20 ml. of saline, filtered through a Seitz filter, and after dilution tested as an immunizing agent.

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† Difco neopeptone 1%, dextrose 2%, agar 2%, pH 6.8.

Animals

Female Swiss white mice weighing 17–19 grams, and obtained from the Animal Production Section of the National Institutes of Health, were used for this investigation.

Male albino rabbits weighing 2.5–3 kilograms, from the above-mentioned source, were utilized for the production of *S. schenckii* antiserum.

Immunization Procedures

Two rabbits were immunized with viable yeast-cell suspensions of *S. schenckii* and two with viable conidiospore preparations. Ten injections of the cells or spores were given intravenously at 5-day intervals with initial doses of approximately 5×10^5 cells, and increasing to approximately 3×10^7 cells at the final administration. The immunizing suspensions were freshly made up for each injection. The animals were test bled 12 days after completion of the schedule for immunization. Agglutination titers of the immune sera ranged from dilutions of 1:1280 to 1:5120, while the preimmunization sera showed no agglutination at 1:20. Cross-agglutination with yeast cells and conidiospores between the homologous and heterologous sera demonstrated no apparent differences. Immune serum from the blood of these rabbits was used for subsequent passive immunization experiments.

Serum from mice with chronic infections of 10 weeks was collected and used for passive immunization studies. It showed an agglutination titer of 1:160, whereas, normal mouse serum had no agglutinating titer at 1:20.

For active immunization of mice with killed antigens, the heated *S. schenckii* cells, formalin-treated, or acetone-treated suspensions were injected intra-abdominally every 3 days. Five doses were given starting with 2.5×10^5 cells in the first injection and increasing to 15×10^6 cells in the final injection. The control groups received sterile saline injections on the same schedule. Seven days after the immunization schedule had been completed, the animals were challenged.

Mice receiving the saline-reconstituted acetone extract of *S. schenckii* yeast-like cells were injected every third day for a total of 5 injections. The dose was increased each time as follows: they first received 0.1 ml. of the 1:100 dilution, then 0.2 ml. and 0.3 ml. of the 1:100 dilution, followed by 0.1 and 0.2 ml. of the 1:10 dilution. Seven days after the fifth injection, they were challenged.

Animals receiving the heated homogenates of infected spleens, and non-heated extracts were given 0.1 ml. of a 1:10 dilution of the original homogenate or extract intra-abdominally every fifth day. Five injections were given. Controls were treated with normal spleen homogenates or extracts, or saline in a comparable fashion.

For the superinfection studies, mice were injected intra-abdominally with 5×10^4 viable *S. schenckii* yeast-like cells. Three groups were infected; one 3 weeks before challenge, another 2 weeks before, and the third group 1 week previous to challenge. Control groups for each time interval were given 5×10^4 heat-killed *S. schenckii* cells. One untreated control group was included in the study.

Sera from the immune rabbits, normal rabbits, infected mice, and normal mice were heated at 56° C. for 30 minutes and diluted 1:20. One tenth ml. was given intra-abdominally to the proper group of mice 24 hours before challenge, 3 hours after, and every other day until the experiment was terminated. The saline controls were injected similarly.

Quantitation of Challenging Doses and Whole Cell Vaccines

The number of *S. schenckii* yeast-like cells for challenging purposes, and immunizing doses of whole-cell vaccines, was determined by direct count in a Levy hemocytometer counting chamber. Tenfold dilutions in sterile 0.85% NaCl solution were made and 0.2 ml. of the appropriate dilution was injected intravenously into the tail vein of the experimental mouse. Thermal dilatation of the veins of the test animals facilitated injection.

Criterion for Interpretation of Results

The endpoint utilized for evaluation of the results being reported was death of the test animals. Mice injected intravenously with *S. schenckii* cells died at a uniform rate ranging from 10 days to 5 weeks depending upon the size of the challenging dose. The survivors were infected and these data represent a comparison of survivor numbers and deaths. All experiments, unless otherwise indicated, were terminated 2 months after challenge.

EXPERIMENTAL RESULTS

Non-Viable Suspensions of Yeast-Like Cells

The data obtained from mice injected with heat-killed suspensions of *S. schenckii* yeast-like cells, and subsequently challenged with viable suspensions, are presented in Table 1. It is obvious that no protection was demonstrated in the treated animals.

The effects of immunization with acetone or formalin-treated antigens are shown in Table 2.

Under the circumstances of this experiment, it appears that the formalin-treated suspensions conferred some protection to the recipient mice. The animals that survived the experimental period of 8 weeks were closely observed at the termination of the experiment. Cultures were made from the livers and spleens of only those groups receiving 3.8×10^2 cells, and since all the other survivors demonstrated external lesions due to sporotrichosis, they were not examined. The livers and spleens of mice receiving the acetone-treated suspension were covered with abscesses and were heavily positive by culture. Cultures were positive from 2 of 5 animals from the group immunized with the formalin-treated suspension, while only 1 of the saline controls was positive. These results suggest that although the death rate was reduced in mice receiving the formalin-treated vaccine, the survivors possessed no apparent superiority in eliminating the infection.

Since the results obtained from the immunization of mice with formalin-treated *S. schenckii* cells were encouraging, another experiment was employed to evaluate the level of immunity in another manner. Separate groups of mice were immunized with a formalin-killed vaccine, acetone-killed cells, the acetone extract from acetone-treated cells, and a control group was injected with saline. After the immunizing schedule was completed, each animal was challenged with 10^6 *S. schenckii* cells. The data from this experiment are shown in Table 3. Under the conditions of this experiment, the level of protection in the group of mice immunized with the formalinized suspension was not as high as expected.

TABLE 1

Deaths of mice vaccinated with heat-killed S. schenckii, *after challenge*

Vaccine	Challenging Dose				
	2.2×10^6	2.2×10^5	2.2×10^4	2.2×10^3	2.2×10^2
Heated cells	5/5	5/5	1/5	0/5	0/5
Saline	4/5	5/5	2/5	0/5	0/5

Numerator indicates number of deaths; denominator indicates number of challenged.

TABLE 2

Deaths of mice vaccinated with an acetone-treated suspension or formalinized suspension of S. schenckii, *after challenge*

Vaccine	Challenging Dose				
	3.8×10^6	3.8×10^5	3.8×10^4	3.8×10^3	3.8×10^2
Acetone treated	4/5	5/5	2/5	0/5	0/5
Formalin treated	2/5	0/5	0/5	1/5	0/5
Saline	5/5	3/5	2/5	0/5	0/5

Numerator indicates number of deaths; denominator indicates number of challenged.

Spleen Homogenates and Extracts

Table 4 presents the results from the immunization of mice with heated homogenates of spleens from infected or normal animals. Very little difference can be observed between the test and control groups. Similar results were obtained using unheated, filtered extracts as shown in Table 5.

Superinfections

The survivor rate was slightly greater in the previously infected groups than in normal mice or those that received the heated yeast-like suspension. The results are presented in Table 6. The length of the initial infection seemed to have little effect upon the survivor rate after challenge.

Passive Immunization

Even though the sera were injected over a period of 8 weeks, no protection was observed in

TABLE 3

Deaths of vaccinated mice after challenge with approximately 10^6 cells S. schenckii

Formalin Treated Vaccine		Acetone Treated Vaccine		Acetone Extract		Saline	
Deaths	% Survivors	Deaths	% Survivors	Deaths	% Survivors	Deaths	% Survivors
39/50	22	45/48	6.2	42.48	12.5	43/50	14

Numerator indicates number of deaths; denominator indicates number challenged.

the groups that received either the immune rabbit serum, or the serum from chronically infected mice (Table 7). No apparent evidence of hypersensitive complications appeared in the surviving mice that received the rabbit serum.

All of the experiments presented in this paper, with one exception, have been repeated with similar results. The exception is the study of immunization with unheated splenic extracts.

It is of interest to note that in the groups of mice which received the acetone-treated *S. schenckii* cell suspension, there was a slight tendency for more susceptibility. This tendency was not manifested by a greater death rate, as shown in Table 2, but by more frequent recovery of the fungus in cultures from the autopsied animals. Table 3 shows more survivors in the control group than in the group receiving acetone-treated cells, but the difference is very small.

TABLE 4

*Deaths in mice vaccinated with homogenates of heated infected spleens or homogenates of heated normal spleens, after challenge**

Vaccine	Challenging Dose				
	7×10^6	7×10^5	7×10^4	7×10^3	7×10^2
Homogenate of infected spleen	5/5	5/5	4/5	0/5	0/5
Homogenate of normal spleen	5/5	5/5	4/4	2/5	0/5
Saline	5/5	4/5	5/5	2/5	0/5

* Experiment terminated 6 weeks after challenge.

Numerator indicates number of deaths; denominator indicates number challenged.

TABLE 5

*Deaths in mice vaccinated with filtered extracts of infected spleens or filtered extracts of normal spleens, after challenge**

Vaccine	Challenging Dose				
	1.9×10^6	1.9×10^5	1.9×10^4	1.9×10^3	1.9×10^2
Extract of infected spleen	5/5	5/5	2/5	0/5	0/5
Extract of normal spleen	5/5	5/5	3/5	0/5	0/5
Saline	5/5	5/5	5/5	0/5	0/5

* Experiment terminated 6 weeks after challenge.

Numerator indicates number of deaths; denominator indicates number challenged.

TABLE 6

Deaths of mice with preinfection or a single injection of heated vaccine, after challenge

Length of Infection or Time of Vaccination Before Challenge	Challenging Dose				
	1.2×10^6	1.2×10^5	1.2×10^4	1.2×10^3	1.2×10^2
Infected 3 weeks	9/10	1/10	1/10	1/10	0/10
Vaccinated 3 weeks	10/10	9/10	5/10	1/10	1/10
Infected 2 weeks	4/10	5/10	1/10	1/10	0/10
Vaccinated 2 weeks	10/10	7/10	1/10	0/10	0/10
Infected 1 week	9/10	1/10	0/10	0/10	0/10
Vaccinated 1 week	9/10	9/10	5/10	0/10	0/10
Saline controls	10/10	6/10	1/10	0/10	0/10

Numerator indicates number of deaths.
Denominator indicates number challenged.

TABLE 7

Deaths of mice receiving high titered antiserum to S. schenckii *from rabbits or serum from infected mice, after challenge*

Source of Serum	Challenging Dose				
	3.5×10^6	3.5×10^5	3.5×10^4	3.5×10^3	3.5×10^2
Infected mice	5/5	5/5	4/5	0/5	0/5
Normal mice	5/5	5/5	0/5	0/5	0/5
Immune rabbits	5/5	4/5	3/5	1/5	0/5
Normal rabbits	5/5	5/5	1/5	0/5	0/5
Saline controls	5/5	5/5	4/5	0/5	0/5

Numerator indicates number of deaths; denominator indicates number challenged.

DISCUSSION

It is apparent from the studies presented here that the protection in mice against sporotrichosis due to vaccination with formalin-treated vaccines or preinfection is of a low degree. Furthermore, considering the methods used to date, it is probably unrealistic to employ the high level of immunity that can be produced to certain bacterial or viral diseases as a base line, when equating artificially induced resistance to the deep-seated mycoses.

Friedman and Smith (1) have shown that killed arthrospores of *Coccidioides immitis* protected mice against lethal doses of viable arthrospores, but that the survivors were infected. Studies by Vogel, *et al.* (2), using *C. immitis* spherules cultured from chick embryos for sensitizing guinea pigs, indicated that less extensive disease was observed in the test animals than in the controls. Investigating artificially acquired immunity to histoplasmosis in mice, Salvin (4) reported a high level of protection by immunizing with formalin-killed acetone dried *Histoplasma capsulatum* yeast cells. Rowley and Huber (3), using a different strain of *H. capsulatum*, were unable to confirm Salvin's results, but presented evidence that preinfected mice were more resistant to challenge than the controls. Later work by Salvin (8) indicated that *H. capsulatum* did not multiply in the tissues of immunized mice at the same rate as in the controls. The course of histoplasmosis (3) in mice was not noticeably altered or modified by the injection of immune serum. Our studies with sporotrichosis gave similar results.

The investigation presented here is in accord with that of Jessner (6) who was studying cutaneous reinfection with sporotrichosis.

The onset of the deep-seated mycoses may be insidious, and they are chronic diseases under most circumstances. It is quite obvious that the laboratory induced resistance to these mycoses does not measure up to the classic immunological criteria applied to many other host-parasite relationships. However, it is logical to consider that the subtle differences of resistance demonstrated by this and other studies derived from relatively short-term experiments, may not illustrate the true importance of even a low level of immunity. This partial immunity may prove to be more significant when extended over longer periods of time.

SUMMARY

Evidence of slight protection of mice against sporotrichosis following immunization with formalin-killed suspensions of *Sporotrichum schenckii*, or preinfection has been presented. Attempts at passive immunization, with serum from rabbits immunized against *S. schenckii* or with serum from infected mice, did not modify the course of the infection.

Interpretation of the results was based on survivor numbers, since all animals were still infected at the end of the experimental period.

A discussion of the possible value of the relatively low level of immunity in this, and in reported studies of other systemic mycoses, is presented.

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