EFFECT OF VACCINES PREPARED FROM *HISTOPLASMA CAPSULATUM* AND OTHER YEASTS ON EXPERIMENTAL TUBERCULOSIS

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ABSTRACT

HEDGECOCK, LOYD W. (Veterans Administration Hospital, Kansas City, Mo.). Effect of vaccines prepared from *Histoplasma capsulatum* and other yeasts on experimental tuberculosis. J. Bacteriol. **82**:115–123. 1961.—Resistance to experimental tuberculosis was enhanced by the injection of a nonviable vaccine prepared from *Histoplasma capsulatum* in the mycelial phase but not by the organism in the yeast phase. Acquired resistance was established within 7 days or less and usually maintained for at least 29 days. When both mycelium vaccine of *H. capsulatum* and a vaccine prepared from tubercle bacilli were utilized in vaccination, the results were additive (in terms of acquired resistance) provided that an interval of 3 weeks was maintained between injection of each of the vaccines.

Resistance to tuberculosis was also demonstrable 14 days after the injection of nonviable preparations of *Brucella abortus* and *Cryptococcus neoformans*, as well as by yeast and mycelial vaccines of *Blastomyces dermatitidis* and *Sporotrichum schenckii*. Resistance decreased significantly when the animals were challenged 29 days after vaccination, with the exception of those injected with the yeast phase of *B. dermatitidis*.

It is established that the injection of nonviable, homologous vaccines stimulates the development of acquired resistance to tuberculosis (Weiss, 1959). Injection of viable and nonviable suspensions of certain heterologous organisms also results in the development of resistance to tuberculous disease. Resistance to tuberculosis was enhanced by the injection of nonviable suspensions of *Hemophilus pertussis*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*, as well as by injection of lipopolysaccharide prepared from gram-negative organisms (Dubos and Schaedler, 1956, 1957). An infection with *Brucella abortus* rendered animals resistant to subsequent infection with *Mycobacterium tuberculosis* (Nyka, 1956).

The present investigation demonstrates that the injection of a mycelial suspension of nonviable *Histoplasma capsulatum* and other pathogenic fungi enhances the resistance of mice to tuberculosis.

MATERIALS AND METHODS

Animals. Female CF_1 mice weighing 14 to 16 g each were housed in groups of 15 to 20 in metal cages. The cages and litter were changed at least weekly. The water was changed on alternate days using sterilized bottles. The stock ration was fed in pellet form, whereas the defined ration was provided in metal feeders (Rusch, Potter, and Miller, 1946).

Diets. Rockland mouse diet represented the standard ration. A casein-supplemented defined ration containing a specified fatty acid mixture, found previously to allow optimal development of resistance to tuberculosis, was also used (Hedgecock 1955, 1958). All rations were furnished ad libitum.

Organisms. M. tuberculosis strain H37Rv, grown in Kirchner medium containing Tween 80 (polyoxyethylene sorbitan monooleate) and bovine albumin (fraction V), was stored in the refrigerator at 5 C. The initial subculture for each experiment was made in the above medium. The second 5-day culture was made in Kirchner medium containing only Tween 80. Cultures of these organisms were used to prepare nonviable vaccines and to infect the animals.

H. capsulatum strain 105 was furnished by R. L. Mayer, CIBA Pharmaceutical Laboratory; it was cultured on Sabouraud medium and stored in the refrigerator at 5 C. For use in initial experiments transfers were made from Sabouraud medium to blood agar slants which were incubated at 37 C for 6 to 8 days. Under these conditions the culture was composed largely of pseudo-

mycelia. With continued transfer and cultivation of this medium at 37 C, the fungus was maintained in the yeast phase. Frequent transfers were necessary to maintain viability. After the observation that the strain could be grown in trypticase soy broth (BBL) in either the mycelial or the yeast phase, depending upon whether the incubation temperature was 22 or 37 C (Garrison, 1960), this medium was employed for propagation. The cultures were agitated during growth. In the case of mycelial cultures, glass beads were included in the culture flasks.

B. dermatitidis CDC strain 27 and *C. neoformans* CDC strain A-645 were cultured in trypticase soy agar or trypticase soy broth (BBL). Culture of *B. dermatitidis* at 37 C produced the yeast phase, but at 22 to 25 C, the mycelial phase developed.

The strain of *Sporotrichum schenckii* utilized was isolated from a patient (Post et al., 1958). Upon cultivation in trypticase soy broth the organism grew in the yeast phase at 37 C and in the mycelial phase at 22 C.

B. abortus strain 19, was obtained as a commercial viable suspension from the Colorado Serum Company, Denver. Cells were washed once prior to preparation of the nonviable vaccine.

Preparation of the nonviable vaccines. All organisms were killed with ethylene oxide as described previously (Hedgecock, 1958). Prior to treatment the cultures of yeast were grown for 6 to 8 days in trypticase soy broth, then washed three times in physiological saline. *B. abortus* was washed once but *M. tuberculosis* was treated without washing. In the procedure the organisms were cooled in an ice bath. One per cent ethylene oxide in the liquid phase was then added. After exposure at this temperature for 1 hr, the organisms were placed in the incubator and held at 37 C for 24 hr. Sterility of the treated cells was determined by inoculation on an appropriate medium.

Immunization and infection of animals. For use as a vaccine, the nonviable suspension of *M. tuberculosis* was diluted in Kirchner medium containing Tween 80 to a transmittance of 70% using a Rouy photometer fitted with a 640 mμ filter. An additional dilution of 1:10 in Kirchner medium was then made and each mouse was injected intraperitoneally with 0.2 ml thereof. In the case of the other organisms, dilutions were made in saline to a transmittance of 70%. Due to variation in the size of the particles, the densities of suspensions of mycelium were not strictly comparable on the basis of light transmittance, with suspensions of bacteria or yeast. But light transmittance could be used as a reference for comparing the densities of different mycelial suspensions. Mycelial suspensions with a light transmittance of 70% were found to contain approximately 0.075 mg of mycelium per ml (dry weight). Except in the cases where varied concentrations of the vaccine were tested or where the effect of multiple injections was determined, 0.2 ml of this dilution was injected intraperitoneally into each mouse.

At periods varying from 7 to 42 days after immunization, each animal was infected by intravenous injection of 0.2 ml of a 5-day culture of strain H37Rv, diluted to 60% transmittance; subsequent deaths were recorded daily. Comparison of different experimental groups was made in terms of the percentage of mice in each group which survived 30 days (S-30) according to the procedure of Youmans and Youmans (1957).

Owing to the large number of animals used, it was not possible to perform autopsies or cultural tests routinely to confirm the cause of death. However, uninfected animals maintained in the same area and under similar conditions remained healthy and free from infection. There was no evidence of Salmonella or other nonmycobacterial infections throughout the animal colony during the studies.

RESULTS

Effect of vaccination with nonviable preparations of H. capsulatum and M. tuberculosis on survival of tuberculous mice maintained on the defined ration. Initially an experiment was designed to determine if the administration of a vaccine prepared from *H. capsulatum* would offer any degree of protection against experimental tuberculosis. In this experiment the vaccine (composed almost entirely of pseudomycelia) was prepared from *H. capsulatum* which had grown for 6 days at 37 C on cystine blood agar after transfer from the stock culture on Sabouraud's agar. The first group of 20 animals was not vaccinated, whereas the second group of 20 was vaccinated with a nonviable autogenous vaccine prepared from *M. tuberculosis*. A third group of 10 animals was vaccinated with *H. capsulatum*. A fourth group of 10 mice was vaccinated with 0.2 ml of a mixture composed of equal parts of the *M. tuberculosis* and *H. capsulatum* vaccines. All animals

were placed on the defined ration at the time of vaccination. Fourteen days after vaccination all animals were infected with *M. tuberculosis*. Fifty-five per cent of the animals in the unvaccinated control group survived for 60 days after infection. This survival value corresponds to that usually observed when the mice are fed defined chow. All of the animals in the groups which had been vaccinated with *M. tuberculosis* and with a mixture of *M. tuberculosis* and *H. capsulatum* survived, whereas 90% of the animals vaccinated with only *H. capsulatum* survived for the experimental period. The findings indicated that vaccination with a nonviable, pseudomycelium preparation of *H. capsulatum* enhanced the resistance of mice to tuberculosis and, furthermore, that the injection of *H. capsulatum* and *M. tuberculosis* simultaneously was not incompatible with the development of acquired resistance to tuberculosis.

A similar experiment was performed in which new vaccine preparations of *M. tuberculosis* and *H. capsulatum* were used and the immunization period prior to infection was extended to 28 days. *H. capsulatum* was prepared from 6-day cultures in trypticase soy broth incubated at 22 C. In this experiment the first and second groups of animals which served as controls were not immunized and were fed chow and defined ration, respectively. The third group of animals was immunized with *M. tuberculosis* and the fourth group with *H. capsulatum*. These animals were maintained on the defined ration. After infection with *M. tuberculosis* the deaths of the animals were recorded daily for 40 days. The S-30 values for groups 1, 2, 3, and 4 were 30, 67, 100, and 89%, respectively. The results of this experiment in which the animals were fed the defined ration were similar to those of the previous experiment in that the *H. capsulatum* vaccine appeared to enhance the resistance of the mice to tuberculosis; however, the resistance was inferior to that effected by the homologous vaccine. There was a significant difference between the rate of death of the unvaccinated animals fed chow and those fed the defined ration. The more rapid development of a high degree of resistance to tuberculosis in mice fed the defined ration as compared to commercial chow has been reported previously (Hedgecock, 1958).

Effect of vaccination with nonviable preparations of H. capsulatum and M. tuberculosis on survival of tuberculous mice fed commercial chow. Since it had been found that vaccination with *H. capsulatum* enhanced resistance to tuberculosis in mice fed the defined ration, a study was made of the effectiveness of the vaccine in mice fed commercial chow. Mice maintained on chow were segregated into three groups. The first group of 15 mice was not vaccinated. The second and third groups of 16 and 14 mice were vaccinated with *M. tuberculosis* and *H. capsulatum* (pseudomycelium), respectively. Eighteen days after vaccination all animals were infected with *M. tuberculosis* and the deaths recorded daily for 83 days. Twenty-seven per cent of the control animals survived beyond 30 days, whereas 50% of the mice vaccinated with *M. tuberculosis* and 72% of the mice vaccinated with *H. capsulatum* survived for an equivalent period. Since *H. capsulatum* was superior to *M. tuberculosis* in enhancing the resistance of mice maintained on chow, it would appear that the vaccine response to *H. capsulatum* was affected less by the dietary action of commercial chow.

A determination was also made of the effect of single and double injections of nonviable suspensions of *H. capsulatum* and *M. tuberculosis* on the development of tuberculosis in groups of mice (15 each) maintained on chow. One group of animals served as the unvaccinated control. Groups 2 and 3 received an injection of a vaccine prepared from *M. tuberculosis*, 20 days prior to infection. Group 3 received an additional injection of vaccine 7 days prior to infection with *M. tuberculosis*. The vaccination of animal groups 4 and 5 was effected as in groups 2 and 3, respectively, except that in this case the former received a vaccine prepared from *H. capsulatum*. Animal group 6 received an initial injection of *M. tuberculosis* vaccine, followed 13 days later by the vaccine composed of *H. capsulatum*. At the end of the 20-day period of vaccination, all animals were infected with *M. tuberculosis*. The S-30 values for animal groups 1, 2, 3, 4, 5, and 6 were 7, 33, 80, 54, 25, and 47%, respectively. Only one animal in the unimmunized control group survived beyond 30 days. A second injection of the vaccine prepared from *M. tuberculosis* resulted in an increase in the S-30 of the infected animals from 33 to 80%. On the other hand, a second injection of the *H. capsulatum* vaccine effected a decrease in the S-30 from 54 to 25%. The decrease in resistance which followed the second injection of *H. capsulatum* vaccine may have resulted from a depressive action of the excessive dose of vaccine

on the agencies responsible for resistance to tuberculosis. The resistance to tuberculous infection which resulted from an injection of the homologous vaccine followed 13 days later by an injection of heterologous vaccine was not equal to that obtained from two doses of the homologous vaccine.

Comparison of the efficacy of vaccines prepared from yeast phase and from mycelial phase of H. capsulatum in experimental tuberculosis. A vaccine was prepared from *H. capsulatum* which had been cultured repeatedly on cystine blood agar at 37 C at 5-day intervals to establish and maintain the yeast phase. The vaccine was diluted over a 125-fold range being injected into four groups of mice in 0.2-ml doses of suspensions with light transmittance of 17, 70, 95, and 99%. A mycelial phase vaccine, prepared from *H. capsulatum* grown for 6 days at 22 C in trypticase soy broth, was diluted and injected into an additional four groups of mice at the above levels. A homologous vaccine prepared from *M. tuberculosis* was injected into a group of mice and another group of animals was retained as an unvaccinated control. During the period of vaccination there was no evidence of toxicity in any of the animals which had received the large dose of either the yeast or mycelial vaccine. On the contrary, the general appearance of the animals was excellent. All mice were infected with *M. tuberculosis* 20 days after injection of the vaccines. Thirty per cent of the unvaccinated mice survived beyond 30 days. The S-30 value of the animals vaccinated with the H37Rv strain was 50%. The four groups of mice which had been vaccinated with the yeast phase preparation of *H. capsulatum* all exhibited S-30 values less than that of the unimmunized control (S-30 values in order of decreasing amounts of vaccine were 20, 18, 0, and 15%). The S-30 values of the animals vaccinated with the mycelial phase of *H. capsulatum* in similar order were 60, 65, 75, and 84%. In terms of survival beyond 30 days, the smallest dose of vaccine afforded maximal resistance. However, when the average mortality time of each group is examined it is apparent that the group of animals which was vaccinated with 0.2 ml of the heterologous vaccine having a transmittance of 70%, acquired the highest degree of resistance to tuberculosis.

Comparison of vaccines prepared from M. tuberculosis and H. capsulatum with vaccines prepared from other pathogenic yeasts. Vaccines were prepared from *B. dermatitidis*, *C. neoformans*, and *S. schenckii* cultured both at 22 C and at 37 C in trypticase soy broth. A nonviable vaccine was also prepared from a commercial preparation of viable *B. abortus*. Groups of 15 mice each were vaccinated with each of the above organisms, or vaccinated in single and double dosage with new preparations of *M. tuberculosis* and *H. capsulatum* (mycelial phase) as well as with a mixture of equal amounts of the two organisms. In addition, mice which were vaccinated initially with the homologous vaccine were again vaccinated 7 days later with the heterologous vaccine. In other groups the initial vaccination was made with the heterologous vaccine and the second injection with the homologous vaccine. Fourteen days after the initial vaccination, each animal was infected with *M. tuberculosis*. The subsequent deaths were recorded daily (Table 1). Thirteen per cent of the unimmunized control animals survived beyond 30 days. A second injection of the vaccine prepared from *M. tuberculosis* effected an increase in the S-30 from 60 to 86.5%. Only 6% of the group of animals vaccinated with a single dose of *H. capsulatum* survived beyond 30 days, whereas 60% of the mice which received two injections of this preparation survived beyond this period. The administration of vaccines prepared from *M. tuberculosis* and *H. capsulatum* simultaneously or in divided dosage did not effect resistance greater than that obtained from a single dose of the former vaccine. These data indicate that this preparation of *H. capsulatum* was relatively impotent as a vaccine. The group of mice vaccinated with *B. dermatitidis* grown at 37 C (yeast phase) died at the same rate as the unvaccinated control group. The S-30 values of the groups of animals vaccinated with each of the other heterologous vaccine preparations including *B. abortus*, were similar to that obtained with a single injection of the homologous vaccine.

The previous experiment was repeated using new vaccine preparations of *M. tuberculosis* and *H. capsulatum*. The vaccine preparations of *B. dermatitidis*, *C. neoformans*, *S. schenckii*, and *B. abortus* were those utilized in the previous experiment. In this experiment the initial vaccination was made 28 days prior to infection. When a second dose of vaccine was required, the injection was made 7 days prior to infection. The deaths of the animals following infection with *M. tuber-*

TABLE 1. *Effect of vaccination with nonviable suspensions of Histoplasma capsulatum in the mycelial phase and of other pathogenic yeasts on survival of tuberculous mice*

Immunizing agent	No. injections vaccine*	No. of mice†	Survival times of mice 60 days after challenging infection	S-30
			days	%
None	0	15	13, 15, 19, 20, 20, 20, 22, 23, 23, 24, 25, 26, 28, 32, 33	13.3
Mycobacterium tuberculosis	1	15	24, 24, 26, 27, 29, 29, 32, 37, 50, 54, S, S, S, S, S	60
M. tuberculosis	2	15	22, 22, 31, 34, 34, 38, 47, 57, S, S, S, S, S, S, S	86.5
H. capsulatum	1	15	15, 17, 19, 19, 20, 21, 21, 22, 22, 24, 25, 25, 26, 28, 33	6
H. capsulatum	2	15	23, 24, 25, 25, 26, 28, 30, 31, 32, 42, 57, S, S, S, S	60
H. capsulatum and *M. tuberculosis*	1	15	20, 21, 22, 23, 23, 24, 25, 28, 39, 51, 52, 53, S, S, S	46.7
H. capsulatum (1st) *M. tuberculosis* (2nd)	2	15	23, 26, 26, 28, 30, 30, 37, 49, 50, 51, 54, 55, 58, S, S	60
M. tuberculosis (1st) *H. capsulatum* (2nd)	2	15	17, 25, 25, 25, 26, 28, 31, 39, 40, 56, S, S, S, S, S	60
Blastomyces dermatitidis (yeast)	1	15	21, 21, 21, 23, 23, 23, 23, 24, 26, 28, 29, 30, 30, S, S	13.3
B. dermatitidis (mycelium)	1	15	22, 22, 24, 27, 27, 27, 29, 33, 36, 48, 48, 52, S, S, S	53.3
Cryptococcus neoformans (yeast at 37 C)	1	15	22, 22, 22, 23, 30, 31, 31, 33, 39, 41, 42, 44, 51, S, S	66.4
C. neoformans (yeast at 22 C)	1	15	24, 24, 24, 25, 25, 27, 34, 41, 47, 57, 60, S, S, S, S	53.3
Sporotrichum schenckii (yeast)	1	15	21, 23, 23, 24, 25, 25, 26, 30, 30, 39, 46, 55, 59, S, S	53
S. schenckii (mycelial)	1	14	17, 21, 22, 22, 23, 24, 25, 31, 36, 38, 49, 50, S, S	50
Brucella abortus	1	15	22, 22, 23, 24, 24, 25, 28, 32, 33, 37, 39, 50, 53, 59, S	53.3

* Initial injection of vaccine administered at 14 days and subsequent (when administered) at 7 days prior to infection with *M. tuberculosis*.

† Animals were fed commercial chow.

culosis are presented in Table 2. All of the unimmunized control animals died within 30 days. A second injection of the *M. tuberculosis* vaccine resulted in an increase in the S-30 of the infected animals from 34 to 85%. On the other hand, a second injection of the vaccine prepared from *H. capsulatum* effected a decrease in the S-30 from 70 to 35%. A single dose of the heterologous vaccine effected greater resistance to tuberculosis than a single dose of the homologous vaccine. These results differ from those obtained when the animals were fed the defined ration which provides conditions suitable for an optimal response of the host to the homologous vaccine. In this experiment the administration of *M. tuberculosis* and *H. capsulatum* either simultaneously or in divided doses enhanced resistance to a degree similar to that produced by two doses of the homologous vaccine. The mice which had been vaccinated with the vaccines prepared from *B. dermatitidis*, *C. neoformans*, *S. schenckii*, and *B. abortus* displayed considerably less resistance to tuberculosis than observed in the previous experiment. Since the immunization period had been increased from 14 to 28 days, an initially high degree of resistance effected by vaccination could have declined to a lower level at the time of infection of the mice.

Effect of multiple injections of nonviable H. capsulatum on survival of mice subsequently infected with M. tuberculosis. In previous experiments a second injection of the mycelial vaccine prepared from *H. capsulatum* resulted in a reduction in the degree of resistance established by the

TABLE 2. *Effect of vaccination with nonviable suspensions of Histoplasma capsulatum in the mycelial phase and of other pathogenic yeasts on survival of tuberculous mice*

Immunizing agent	No. injections vaccine*	No. of mice†	Survival times of mice 136 days after challenging infection	S-30
			days	%
None	0	20	5, 13, 14, 14, 15, 17, 17, 17, 19, 19, 19, 20, 20, 20, 21, 21, 21, 23, 23, 25	0
Mycobacterium tuberculosis	1	18	8, 14, 19, 19, 19, 20, 21, 21, 21, 22, 23, 26, 37, 50, 56, 77, 100, 112	33.8
M. tuberculosis	2	20	18, 28, 29, 32, 49, 50, 54, 70, 81, 85, 88, 90, 90, 90, 94, 98, 100, 102, 116, S	85
H. capsulatum	1	20	21, 22, 22, 23, 23, 23, 35, 35, 35, 42, 45, 45, 54, 59, 65, 77, 78, 83, 85, 86	70
H. capsulatum	2	20	18, 18, 19, 19, 19, 20, 21, 21, 21, 21, 23, 24, 24, 32, 35, 37, 70, 81, 81, 83	35
H. capsulatum and *M. tuberculosis*	1	20	21, 22, 25, 25, 26, 27, 49, 49, 50, 68, 70, 71, 72, 78, 86, 86, 88, 90, 94, 107	70
H. capsulatum (1st) *M. tuberculosis* (2nd)	2	20	20, 25, 38, 45, 57, 58, 60, 60, 64, 65, 68, 70, 70, 81, 82, 88, 102, 102, 127, 128	90
M. tuberculosis (1st) *H. capsulatum* (2nd)	2	20	19, 21, 22, 23, 42, 48, 57, 58, 59, 60, 60, 70, 78, 78, 82, 83, 86, 92, 106, S	80
Blastomyces dermatitidis (yeast)	1	20	18, 18, 18, 19, 19, 19, 20, 21, 21, 23, 23, 24, 25, 28, 51, 60, 69, 81, 85, 87	30
B. dermatitidis (mycelium)	1	20	18, 19, 19, 19, 20, 20, 20, 20, 20, 21, 22, 23, 23, 24, 24, 24, 26, 29, 32, 48	10
Cryptococcus neoformans (yeast at 37 C)	1	20	18, 18, 19, 19, 19, 19, 20, 20, 20, 20, 20, 21, 21, 23, 24, 50, 51, 81, 108, 136	25
C. neoformans (yeast at 22 C)	1	20	13, 14, 14, 17, 21, 21, 22, 22, 23, 24, 25, 31, 33, 33, 52, 57, 65, 65, 81, 100	45
Sporotrichum schenckii (yeast)	1	20	17, 18, 19, 19, 19, 20, 20, 20, 21, 22, 23, 23, 29, 49, 58, 59, 69, 96, 98, 112	35
S. schenckii (mycelium)	1	20	17, 19, 19, 19, 19, 20, 20, 20, 20, 20, 20, 20, 21, 23, 24, 27, 32, 33, 37, 56	20
Brucella abortus	1	20	17, 18, 18, 19, 19, 19, 19, 20, 20, 20, 21, 21, 21, 21, 21, 22, 23, 27, 47, 57	10

* Initial injection of vaccine administered at 28 days and subsequent dose (when administered) at 7 days prior to infection with *M. tuberculosis*.

† Animals were fed commercial chow.

initial single injection. In an effort to obtain additive effects from multiple injections of the *H. capsulatum* vaccine, an experiment was performed in which smaller doses of the vaccine were administered over a longer period of time. In this experiment five groups of animals were utilized. The first group was not immunized. The second group was injected with 0.2 ml of a suspension of *H. capsulatum* diluted to a light transmittance of 70%, at 6 weeks prior to infection with *M. tuberculosis*. Additional injections of the vaccine diluted 1:10 were made at 3, 2, and 1 week prior to infection. The third group received injections of the diluted vaccine at 5, 3, and 1 week before infection, whereas in a fourth group the diluted vaccine was administered at 5 and 2 weeks before challenge with *M. tuberculosis*. Animals of a fifth group were immunized with a single dose of undiluted mycelial vaccine 3 weeks prior to infection. The results are recorded in Table 3. The S-30 value for the unimmunized group of mice was 33%. All of the animals of group 2 which had been immunized by the injection of *H. capsulatum* at 6, 3, 2, and 1 week prior to infection survived beyond 30 days. The S-30 values for animal groups 3, 4, and 5 were 60, 70, and 75%, respectively.

Time required for development of resistance to

TABLE 3. *Resistance to tuberculosis after multiple injections of nonviable vaccine prepared from Histoplasma capsulatum in the mycelial phase*

Group	Immunizing agent	Time of injection of vaccine prior to infection	No. of animals*	Survival time of mice 74 days after challenge with *Mycobacterium tuberculosis*	S-30
		weeks		*days*	%
1	None	None	18	14, 16, 16, 17, 20, 20, 21, 21, 23, 23, 25, 30, 34, 40, 59, 71, S, S	33.3
2	*H. capsulatum*	6†-3-2-1	18	32, 53, 54, 59, S, S, S, S, S, S, S, S, S, S, S, S, S, S	100
3	*H. capsulatum*	5-3-1	20	22, 22, 22, 24, 25, 25, 26, 26, 32, 41, 43, 51, 54, 57, 58, 65, 65, 67, S, S	60
4	*H. capsulatum*	5-2	20	16, 17, 21, 22, 25, 27, 35, 37, 41, 45, 46, 51, 52, S, S, S, S, S, S, S	70
5	*H. capsulatum*	3†	20	21, 22, 22, 23, 24, 32, 35, 37, 38, 39, 49, 50, 51, 51, 58, 68, S, S, S, S	75

* Animals were fed commercial chow.

† The mice were vaccinated with a nonviable suspension of *H. capsulatum* in the mycelial phase exhibiting a light transmission of 70%. In all other injections in the immunization schedule, 0.2 ml of a suspension with a light transmittance of 97% was used.

tuberculosis after injection of vaccines prepared from M. tuberculosis and H. capsulatum. Four groups of mice were vaccinated with *M. tuberculosis* at 7, 14, 22, and 29 days prior to infection. Four other groups of animals were injected at identical times with a vaccine prepared from *H. capsulatum.* An additional group of mice received an injection of a mixture of equal parts of *H. capsulatum* vaccine and a vaccine prepared from a strain of *S. aureus* which had been isolated as a contaminant from a culture of tubercle bacilli. A group of unvaccinated animals served as the control. The animal groups were composed of from 17 to 19 mice each. At the end of the period of vaccination all animals were infected with *M. tuberculosis.* The mice which had been vaccinated with *M. tuberculosis* at 28, 22, 14, and 7 days prior to infection exhibited S-30 values of 68, 53, 44, and 72, respectively. Only 15.8% of the mice vaccinated with *H. capsulatum* 29 days prior to infection survived beyond 30 days, whereas the group of animals which had been vaccinated with a mixture of *H. capsulatum* and *S. aureus* exhibited an S-30 of 47%. The S-30 values of the groups of mice which had been vaccinated with *H. capsulatum* at 7, 14, and 22 days prior to infection were 52, 50 and 63%, respectively. It would appear that the stimulus evoked by the vaccine of *H. capsulatum* had declined to an inadequate level after 29 days but that the inclusion of a second organism in the vaccine (*S. aureus*) provided the added stimulus required for maintenance of a significant amount of resistance at the time of tuberculous infection. It is apparent that resistance to tuberculosis was established within 7 days following the administration of both of the vaccines.

DISCUSSION

It has long been known that the injection of killed bacteria and their products may alter nonspecific resistance of the host to experimental infection (Dreyer and Walker, 1910). Endotoxin (lipopolysaccharide) prepared from a variety of gram-negative organisms and zymosan (Pillemer and Ecker, 1941), an insoluble carbohydrate derived from the cell walls of yeast, represent microbial products which when injected into animals produce a rapid and transient increase in resistance to experimental infection (Evans and Perkins, 1954; Pillemer and Ross, 1955; Rowley, 1956; Dubos and Schaedler, 1956, Landy and Pillemer, 1956). Although the animal is more susceptible immediately after injection of the products, resistance is established 24 to 48 hr later and maintained from 1 to 10 weeks (Dubos and Schaedler, 1957; Rowley, 1956). An endotoxin has been reported to be present in yeast cells of *H. capsulatum* (Salvin, 1952); the mycelial phase of the organism was not examined. There was no evidence of toxicity in our animals after the administration of large doses of yeast and

mycelial vaccine preparations. This finding would indicate that the enhancement of resistance to tuberculosis by the mycelial preparation of *H. capsulatum* is due to a factor other than endotoxin. On the other hand, injection of yeast vaccines prepared from *B. dermatitidis*, *S. schenckii*, and *C. neoformans* did increase resistance to tuberculosis.

There are a number of reports in the literature which demonstrate that the injection of heterologous vaccines and other nonspecific stimuli may further enhance an antibody titer initially established by a homologous vaccine (Freund, 1953). Since *M. tuberculosis* and *H. capsulatum* are considered to be antigenically distinct (Palmer and Edwards, 1960), enhancement of resistance to tuberculosis by a second dose of heterologous vaccine following an initial injection of homologous vaccine would provide an example of "nonspecific anamnestic response." In infections wherein the antibody titer is directly related to the state of resistance of the host, a nonspecific anamnestic response would be expected to play a significant role in the host-parasite relationship. It is possible that an alerting of the reticuloendothelial system by a heterologous vaccine could also result in enhancement in the development of immunity in infections where no correlation between demonstrable antibody levels and resistance has been established.

In a majority of the experiments in which a relatively large dose of vaccine was used, a single dose of *H. capsulatum* mycelial vaccine resulted in the establishment of a significant degree of resistance to tuberculosis, but the injection of a second large dose effected a decrease in resistance, even when the vaccinations were spaced 3 weeks apart. However, when the heterologous vaccine was followed 3 weeks later by the homologous vaccine, additive results were obtained. These results were also obtained in the experiments where the heterologous and homologous vaccines were administered in the reverse order when the same time relationships were observed. When the period of time between injection of heterologous and homologous vaccines was decreased, resistance was never greater than that resulting from a single injection of homologous vaccine. Since in these experiments the dosage of *H. capsulatum* vaccine was quite large (ten times the concentration of *M. tuberculosis* vaccines used) it appeared possible that a second injection of a large dose of the heterologous vaccine over a relatively short period of time effected paralysis of the biological system responsible for acquired resistance to tuberculosis (Felton and Ottinger, 1942). Then the finding that cumulative results were obtained when the heterologous and homologous vaccines were administered alternately might indicate that the active substances in the two vaccines were chemically and biologically different, or the effect might have been related only to the relatively small size of the dose of *M. tuberculosis* vaccine administered.

A cumulative increase in resistance to tuberculosis through multiple injections of *H. capsulatum* mycelial vaccine was accomplished only when a longer period of time was utilized in the vaccination procedure and when the dose of vaccine was reduced. The highest level of resistance to tuberculosis was established when a large dose of *H. capsulatum* vaccine was injected 6 weeks prior to infection, followed by injections of doses of vaccine reduced 10-fold, at 3, 2, and 1 week prior to challenge with *M. tuberculosis*. When the smaller dose was administered throughout on a similar schedule the level of resistance was not significantly greater than that obtained from a single injection of the large dose of mycelial vaccine.

The great difference in rate of death of tuberculous mice fed chow and the defined ration has been reported previously (Hedgecock, 1955; 1958). It was demonstrated that resistance to tuberculosis develops more rapidly and to a higher degree in vaccinated and unvaccinated mice maintained on the defined ration. In the case of the unvaccinated mice, 50 to 80% of the tuberculous animals fed the defined ration acquire sufficient resistance during the early stage of the infection so that the disease followed a chronic rather than an acute course. Current investigations indicate that the enhanced immunological response of the mice maintained on the defined ration is not due to a superior nutritional status but to the presence of a factor (or factors) in the chow which depresses the development of acquired resistance to tuberculosis.

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