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DEVELOPMENT OF VACCINES FOR COCCIDIOIDOMYCOSIS

by

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ABSTRACT

A most compelling stimulus to investigate the influence of actively-induced immunity in experimental coccidioidomycosis is found in the epidemiologic studies of SMITH (35) and SMITH et al. (37—39). Their serologic and skin test surveys among residents in areas of coccidioidal endemicity directed attention to a role apparently played by actively-acquired immunity in man exposed to *Coccidioides immitis*. It was found that after recovery from illness, or after the acquisition of dermal sensitivity to coccidioidin in the absence of manifest illness, there was very little likelihood of a second symptomatic coccidioidal episode. SMITH (personal communication) inferred from the above observation that *C. immitis* was endowed with effective immunogens and speculated (37) that live or killed coccidioidal vaccines could be of profound public health significance. During the two decades following the report of SMITH et al. (39) on the use of coccidioidin, workers explored the efficacies and advantages of live and killed vaccines in experimentally produced coccidioidomycosis in animals.

LIVE VACCINES

Although RIXFORD & GILCHRIST (33) and, later, NEGRONI et al. (27) were the first to demonstrate that experimentally infected animals were more resistant to superinfection, it was SMITH (36) who first showed that mice immunized intraperitoneally with a strain of low virulence developed immunity to lethal intraperitoneal challenge doses of a virulent strain. PAPPAGIANIS et al. (29) confirmed that immunity so-induced was also demonstrable after intranasal challenge but the protective index (ratio of LD_{50} in vaccinated animals /LD_{50} in nonvaccinated) was not high.

Very marked immunity to intranasal challenge doses, however, with immune indices exceeding 4 logarithms, was produced by

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intranasal vaccination with a mutant culture (29). The mutant, designated strain 887, was a riboflavinless avirulent auxotroph (7) derived after X-irradiation of a virulent strain, Silveira. Its potential utility as an immunizing agent for man, however, was rejected by PAPPAGIANIS et al. (29) after it was shown that mice harbored the vaccinating organism in pulmonary lesions for more than 90 days.

The capacity of relatively avirulent strains of *C. immitis* to persist in the host, or to disseminate from the inoculation site to regional nodes and beyond, is the crux of the live vaccine problem. There can be no doubt that the live vaccines are strikingly efficacious in experimental animals. PAPPAGIANIS et al. (30) and CONVERSE et al. (3—6) showed this with live arthrospore vaccines in monkeys. CASTLEBERRY et al. (2) demonstrated it in dogs and FRIEDMAN & SMITH (8), PAPPAGIANIS et al. (29), CONVERSE et al. (3), and others concurred after essentially comparable studies in the mouse. All of these investigations made clear, however, that pathologic processes following inoculation were a matter of concern.

Accordingly, some workers have incorporated procedures to minimize the sequelae of live vaccination by treatment of the host or the vaccinating organism. These have included: 'inactivation' of the vaccinating strain by ^{60}Co irradiation (31) prior vaccination with killed organisms (5) and the administration of amphotericin-B after vaccination (1,2). In large measure such efforts have met with success. However, the nephrotoxicity and limitations of the antibiotic, the possibility of reversion of 'inactivated' organisms to normalcy and the risks inherent in a vaccinating regimen that depends upon the hosts' response to killed cells for its subsequent protection during deliberate infection, present additional problems. But such problems are secondary to yet another which underlies the use of any live vaccine. It is the problem of ascertaining that a vaccinating strain of low virulence will not increase in virulence during its sojourn in the host. KONG & LEVINE (12) investigated this feature in *C. immitis* by determining the extent of virulence loss and recovery that could be induced in strain Silveira.

They observed that a marked reduction in virulence for mice occurred when arthrospores were converted to spherules and endospores by repeated passage in a defined medium. After 84 transfers, the minimum intranasal dose required to kill 20 % of the mice increased from 20 to 370,000 organisms. This loss of virulence was gradual and was evident also when the spherules were reconverted to arthrospores.

The attenuated spherules and endospores, and the arthrospores derived from them, were highly and comparably efficacious as live vaccines. Doses of 5 to 10 thousand endospores given intranasally to mice, produced no apparent illness although the organisms multiplied in the lungs and produced granulomatous lesions containing spherules. The lesions were well circumscribed and, generally, the lungs were cleared of fungus by 31 days after exposure.

However, the virulence of such attenuated organisms could be increased both *in vitro* and *in vivo* After 3 to 5 subcultures on complex agar medium, 10^4 to 10^5 arthrospores were lethal to 25 to 38 % of mice; prior to subculture, these doses killed none. Lung lesions were more severe than previously and did not become free of *C. immitis* within 30 days. After an additional passage *in vivo* all morphologic forms increased further in virulence.

Thus, in the study, attenuated virulence was an unstable property of *C. immitis* and the application of attenuated live vaccines derived from strain Silveira as described, was judged to be hazardous.

Nonliving Vaccines

An important consideration, neglected in early studies on nonviable coccidioidal vaccines, pertains to the micromorphology of *C. immitis*. Whereas it is primarily the spherule-endospore forms of the fungus that parasitizes man, infections are initiated in nature by arthrospores. This relationship poses an immediate question that asks if there are immunologic advantages that may be gained by utilizing vaccines of one form in preference to the other. Concurrently, one may ask if either form possesses immunogenic attributes lacking in the mycelial form, which has the technical advantage that it may be grown easily and with minimum hazard.

Answers to these questions were not apparent from the earlier trials with killed vaccines because the conditions of vaccination and challenge varied markedly. Thus, Vogel et al. (41) showed that vaccination with killed spherules, grown in embryonated eggs (40) caused reduced lesions when guinea pigs were challenged by the respiratory route. Friedman & Smith (8) demonstrated that the administration of killed arthrospores conferred significant resistance to mice challenged intraperitoneally, and Pappagianis et al. (29) confirmed this finding but demonstrated further that many of the vaccinated mice were not resistant to comparable challenge doses given intranasally.

Comparisons under standard conditions were, however, made by Levine, Cobb & Smith (20, 21). The three forms of *C. immitis* were grown *in vitro* standardized by weight, killed with formalin in the cold, and were employed as vaccines at comparable doses in mice. The animals were challenged in parallel with arthrospores and the intranasal route was used for this purpose. The results showed that the spherule-endospore growth formwas immunogenically superior; immune indices in the magnitude of 100 were obtained with the spherule-endospore vaccine compared to approximately 10 with the mycelial or arthrospore preparations. In later studies, Kong & Levine (11) described that an immune index of approximately 1000 could be induced by vaccination with formalin killed spherules. Similarly, the spherule-endospore vaccine was strikingly efficacious in Cynomolgous monkeys challenged by the respiratory route

(25). Seven vaccinated and ten nonvaccinated animals were used in the study. It was found that 5 of the 10 control monkeys died and 2 became moribund within 9 months after infection with 200 arthrospores. None of the 7 vaccinated animals succumbed to this dose but one, challenged with 400 arthrospores, died within 7 weeks. Both early and late roentgenographic changes in the lungs were less extensive in the vaccinated than in the control group and this observation was in accord with the pathologic findings at necropsy. The etiologic agent was recovered from all animals.

In monkeys, therefore, as in mice (20, 21), vaccination did not prevent infection. It sustained life and prevented (in mice) or minimized (in monkeys) the occurrence of disseminated disease and it limited the extent of fungal multiplication in the host (14). The latter feature appeared to reflect an augmented capacity in vaccinated animals to respond at the cellular level to *C. immitis* (34). Typically, the lesions in vaccinated mice and monkeys were fewer and smaller than in nonvaccinated animals and were not as widely distributed. Fewer of these lesions had undergone necrotic degeneration and they contained significantly fewer organisms than those in the nonvaccinated animals. The pulmonary granulomas in the vaccinated groups were superior architecturally to those in the nonvaccinated; the round cell infiltrate was more pronounced and the periphery was comprised of dense fibrous stroma (14).

The possibility of inducing sufficient immunity to enable mice to clear their tissues completely of challenge organisms was investigated by KONG et al. (16). It was accomplished by administering a booster dose of 20 to 200 μg of killed spherules to spherule-vaccinated mice 6 to 7 days before challenge. In this circumstance, after challenge with 100 LD_{50}, fungal proliferation in the lungs was suppressed up to 5 million-fold and 60 to 75 percent of the animals became free of the organism within three months. Clearance activity began sooner in boosted and vaccinated mice than in those that had only been vaccinated, and was paralleled by an earlier rise in the numbers of inflammatory cells in the lungs. Although booster treatment shortly before infection may not be immediately applicable in immunization procedures in the field, the study, nevertheless, emphasized the anamnestic similarity between the immune responses in coccidioidal disease and in certain bacterial, viral and toxic diseases.

Similarly, the immune response to spherules could be impaired by inducing unresponsiveness or immune tolerance in a manner that paralleled its induction in other systems (23) and the attributes of unresponsiveness with *C. immitis* had features resembling those described for other antigens. When small amounts of vaccine, 120 μg or more, were administered intravenously to mice, either concurrently with, before, or up to 35 days after vaccination with spherules by the intramuscular route, the immune response did not develop. The animals succumbed to challenge with 5 LD_{50}

whereas in the absence of intravenous treatment they withstood 200 LD_{50}.

The intravenous administration of antigen impaired immunity development, not its expression, because preimmunized mice were not affected adversely by the treatment. The phenomenon was immunospecific and could be mediated by endospores and the cell wall fraction of mature spherules.

A point of dissimilarity with other systems, however, was the slow rate of immunity development in mice vaccinated with spherules; the response became optimal only after 35 days or more had elapsed. Apparently, the rate was governed by the rate at which immunogens were released catabolically from the highly insoluble, chitinous, spherule walls, as well as the rate of spherule transport from vaccination sites to lymphatic and reticuloendothelial loci. These relationships were inferred by LEVINE & KONG (22) from studies with radioactive spherule walls and studies in which the vaccination sites were removed surgically at intervals after injection.

In the investigations on immune tolerance and the dynamics of development of the immune response, either spherules, their walls, or endospores were employed for different purposes. The immunogenic relationships among these structures had been elucidated by mycological studies and fractionation procedures. Immunogenicity was an attribute of the young naturally-liberated endospore (24). This attribute became more pronounced as the endospores grew in a chemically-defined medium. Concommitant with growth, the structure enlarged and underwent morphologic changes until it had the appearance of a mature spherule. Immunogenicity was then maximal. In contrast, endospores taken from mechanically disrupted spherules were nonimmunogenic (KONG et al., 1963). Apparently, the immunity-inducing antigens were biosynthesized either shortly after or, possibly, shortly before the endospore was released from the naturally-rupturing spherule.

After mechanical disruption of the mature spherule and the separation of components, KONG et al. (13) found that immunogenicity resided almost exclusively in the wall fraction. This observation and those outlined above suggested that the morphogenetic development of the endospore was accompanied by antigenic changes in its wall and these accounted, at least in part, for the spherule's immunogenicity. Other antigenic distinctions between the spherule and mycelial growth forms have been reported recently by LEVINE et al. (19) LANDAY et al. (17, 18) and RAY & CONVERSE (32), and KONG, SAVAGE & KONG (15).

That there is, nevertheless, sufficient antigenic similarity within the genus *Coccidioides* to suggest that a monovalent vaccine may be practicable is inferred from the study of HUPPERT et al. (9). These workers subjected mice vaccinated with strain Silveira spherules to challenge with strains that were markedly atypical in their morphologic, cultural and microscopic properties (10). In all, 5

diverse and two typical strains were employed and the vaccinated mice were well-protected in each instance.

STUDIES ON KILLED SPHERULE VACCINES IN HUMAN BEINGS

The potential efficacy of the spherule vaccine in human beings is unstudied; to date experimentation in man has been confined to determining how well the vaccine is tolerated in man and its influence on serologic and sensitivity responses to coccidioidin.

LEVINE & SMITH (26) determined that intramuscular dosages up to at least 2.7 mg were well-tolerated. An adverse reaction, mild generalized urticaria and tenderness at the vaccination site, was observed in one of eight subjects. The subject suffered no respiratory distress, rhinitis, cough or temperature rise. Inasmuch as the volunteer described himself as hyper-allergic to certain grasses, strawberries, beer, as well as to vaccines received during military service, his mild reaction to spherules does not appear to contraindicate their use.

Six of the volunteers, nonsensitive to coccidioidin prior to vaccination, responded positively in the skin test after treatment. None developed complement fixing or precipitating antibodies. However, one, dermally sensitive to coccidioidin before vaccination, produced complement fixing antibodies after the injection of killed spherules.

PAPPAGIANIS et al. (28) extended these studies in 59 male volunteers in a prison facility in California with two lots of vaccine. At doses below 5 mg, one lot of vaccine was generally well-tolerated, but the second produced local irritation. Occasional mild tenderness and induration at the vaccination site was observed with the first lot. Infrequently, the tenderness and induration were pronounced. At doses greater than 5 mg, there was local discomfort and swelling that usually subsided in 7 to 10 days.

In contrast to the findings of LEVINE & SMITH (26), the induction of sensitivity to coccidioidin was irregular and weak. Transient serologic responses, unrelated to hypersensitivity reactions, were observed in three subjects.

These preliminary studies emphasize a difficulty that can be anticipated in a vaccination trial. Human response in terms of serology and hypersensitivity is variable and, consequently, a demonstrable biological change following vaccination will not be universally available. It should be noted, hower, that such a demonstrable "marker" may not be a prerequisite of immunity. LEVINE, MILLER & SMITH (25) observed that only 3 of 7 monkeys developed dermal sensitivity (induration) after vaccination and none showed serologic changes, yet all were protected against a severe challenge dose.

Two other problems can be anticipated in a trial wherein vaccine efficacy can only be gauged by the frequency of illness among subjects naturally exposed to *C. immitis* in an endemic region. The

first is that only 40 % of the nonvaccinated volunteers can be expected to contract illness and in only approximately half of these can the illness be anticipated to be sufficiently severe to require medical attention. Thus, for the findings to have statistical meaning, the initial number of volunteers would have to be very large. The second problem is that the time of exposure cannot be predicted; it may occur shortly after arrival of a volunteer in an endemic region or only after many years. Hence the clinical followup would have to be long-term. Since both military and civilian populations in this country are mobile, difficulties in followup can be anticipated. The public health advantages to be gained if vaccination was successful, however, argue in favor of a trial.

Summary

In many respects the achievements to date on coccidioidal vaccines is an exciting example of productive collaboration among workers in several biomedical disciplines. Epidemiologic and serologic surveys first showed that a pattern of resistance to *C. immitis* emerged in the natural course of disease and exposure. Immunologists explored the efficacy of inducing such active immunity artificially with live and killed vaccines. And mycologists, immunologists and biochemists cooperated to determine the morphogenetic determinants of immunogen biosynthesis, the locus of immunogens in fungal structures and many of the attributes of induced immunity. These varied studies led to the development of a killed spherule vaccine that was highly efficacious for experimental animals. Whether or not this interdisciplinary effort reaches a fitting climax – successful vaccination in man – remains for the future.

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