BACTERIOLOGICAL REVIEWS, Mar., 1967, p. 35-53
Vol. 31, No. 1

Experimentally Induced Immunity in the Mycoses[1]

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INTRODUCTION

The increased awareness in recent decades of the prevalence of airborne fungi and their high frequency as etiological agents of pulmonary disease has stimulated widespread interest in their pathogenicity and immunogenicity. The chronicity and severity of mycoses produced by *Coccidioides immitis*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Cryptococcus neoformans* pose problems of considerable significance in public health. Of further concern is the susceptibility of certain races to severe coccidioidomycosis and histoplasmosis, and the frequency of intercurrent disease in histoplasmosis and cryptococcosis. On the other hand, epidemiological data also show that man's defense against these organisms is truly remarkable. In histoplasmosis and coccidioidomycosis, the two diseases most extensively studied, infections are often asymptomatic or mild, and immunity following infection is strong (100, 114).

The defense mechanisms of man in the mycoses, however, are little understood. Antibodies, arising during the course of disease, have high diagnostic and prognostic values, but their protective effect, if any, has not been demonstrated. Cellular responses producing granulomas and delayed hypersensitivity reactions are salient features; the former is associated with heightened immunity, but the role of the latter is undetermined.

Studies in animals to elucidate the pathogenesis of fungal diseases and to develop potent vaccines have been hampered by intrinsic and extrinsic factors. The hazards involved in handling pathogenic fungi (especially the spore formers), the large size of the organisms, and their morphological variability introduce difficulties in experimentation. A survey of the literature shows that, in some cases, insufficient attention has been paid to these and other factors which we consider important in experimental fungal immunology. Nevertheless, inroads have been made, and these merit review; enhanced resistance to the above organisms has been achieved by immunization (44, 60, 100), and the site of the immunogens in

[1] A co ntribution to the round table "Respiratory Mycoses: Comparative Epidemiology, Ecology, and Immunology," held at the Annual Meeting of the American Society for Microbiology, Los Angeles, Calif., 3 May 1966, with Roger O. Egeberg as convener.

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C. immitis (55), *H. capsulatum* (103) and, possibly, *C. neoformans* (34) is known. Although the immune response to *B. dermatitidis* is relatively unstudied, in the present review, the findings on induced resistance to all four organisms will be examined together and, wherever possible, compared and contrasted.

General Considerations for Demonstrating Fungal Immunity

Evaluation of the Extent of Immunity

A prerequisite for the demonstration of enhanced resistance is the use of a susceptible host. The host range for *C. immitis*, *H. capsulatum*, *C. neoformans*, and *B. dermatitidis* has been reviewed recently (100, 114). By far the most widely used species is the mouse, and the choice of strain (30), sex (62, 107), and age (107) of this host appears to be important. With *Coccidioides*, the LD_{50} for male mice is lower than that for the female (62), but the extent of protection afforded by vaccination, as determined by displacement of LD_{50}, is approximately the same for both.

The extent of immunity may be evaluated by several criteria which are discussed here only in general terms. Survival after challenge is a popular as well as a stringent criterion, especially when high challenge doses are used. However, in fungal infections, the mortality endpoint generally requires observation periods of several weeks to months, depending on the route and dose of challenge. Observations limited to shorter periods often serve only to demonstrate the capacity of induced immunity to delay death; such resistance may be nonspecific. A sensitive criterion of immunity is the suppression of fungal multiplication; as will be shown in a different section, immunity wane or enhancement may be thus demonstrated. The extent of multiplication may be determined at the inoculation site as well as at the loci of dissemination, and may be correlated further with histopathological findings.

Modifications in the course of localized reinfections have also been used as an index of immunity to histoplasmosis in the eye (20) or ear chamber (3). However, this type of response tends to indicate only the presence, but not the extent, of resistance. Delayed hypersensitivity reactions

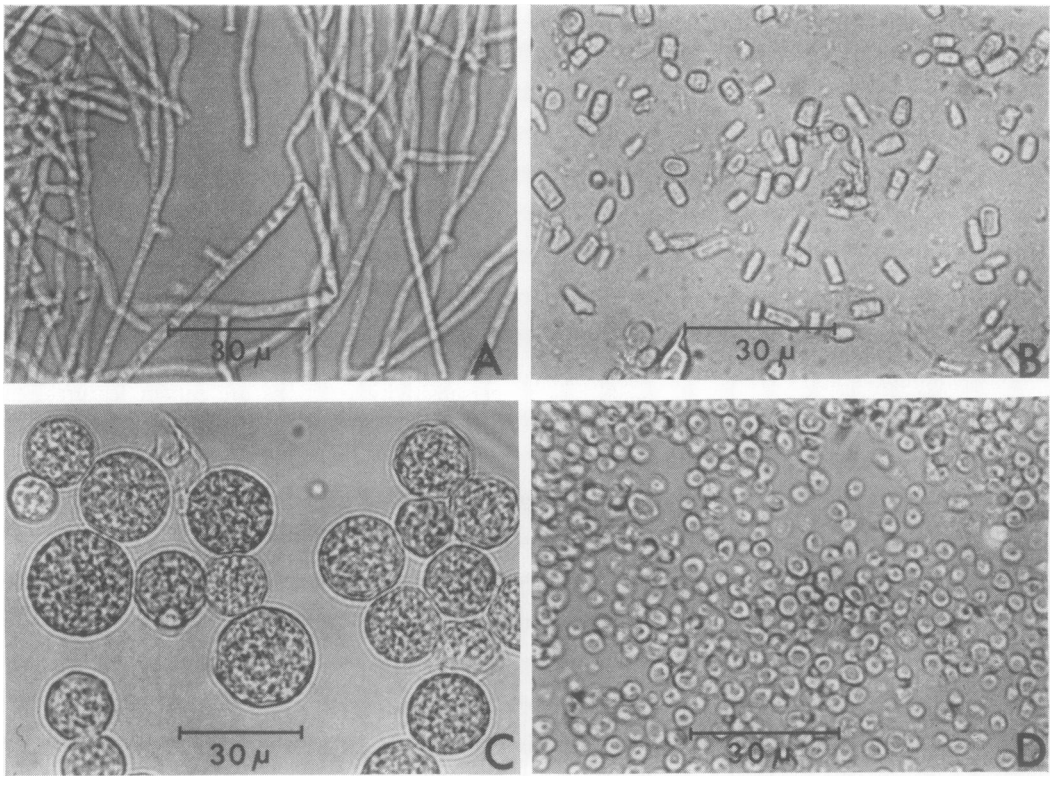

FIG. 1. *Growth phases of Coccidioides immitis strain Silveira: (A) mycelia, (B) arthrospores, (C) spherules, (D) endospores.*

(56, 80, 97) and their development have not been correlated with immunity to challenge, and therefore cannot be used even to indicate the presence of resistance.

Morphological and Cultural Factors

In growing bacteria and viruses, the organisms obtained are generally quite uniform in size and morphology, so long as the medium or tissue culture is appropriate. In contrast, cultures of *C. immitis*, *H. capsulatum*, and *B. dermatitidis* not only vary greatly in size and shape but are often multiform in a given medium. These inherent cultural characteristics of fungi make purification of the growth phases or even determination of fungal numbers difficult to achieve. A notable example is *C. immitis* with four distinct morphological forms (Fig. 1), all of which may be found in a single culture flask. To obtain high yields of spherules and endospores, repeated transfers in a defined medium are required, and removal of the mycelial phase is usually necessary (53, 65).

It is now known that antigenicity varies with the morphological phase or characteristics of certain fungi. In *Cryptococcus*, small capsule strains were more antigenic in rabbits (49, 79) and induced stronger resistance in mice (2) than did large capsule strains. A similar increase in antigenicity was obtained by enzymatically degrading the capsule (33) of a large capsule strain. Absorption of fluorescent antibodies to *H. capsulatum* or *B. dermatitidis* with cross-reacting preparations showed the presence of antigens specific for the yeast phase of each (50). The yeast-phase cells of both fungi are also highly immunogenic (44, 45, 95, 109). Since mycelial culture filtrates of *H. capsulatum* also induced a low order of resistance (94), it is apparent that there are immunogens common to both phases. The qualitative and quantitative relationships of the immunogens, however, are yet unstudied, and it is not known whether the yeast phase contains additional immunogenic determinants.

Marked difference in immunogenicity was observed, however, between the saprophytic and parasitic phases of *C. immitis* (Fig. 1). Mycelia and arthrospores, sterilized with Formalin, protected mice well against low challenge doses by the intraperitoneal route (14, 29, 82) but very poorly against challenge by the intranasal route (82). However, in direct comparisons of the killed saprophytic and tissue forms grown in different media, Levine et al. (61, 62) observed that spherules and endospores afforded stronger protection to mice against intranasal challenge doses than did mycelia and arthrospores. Moreover, using mycelia and spherules grown simultaneously in the same flasks of medium, we have recently observed that spherule-vaccinated mice were protected against a challenge of approximately 200 LD_{50} compared with approximately 20 LD_{50} in mycelia-vaccinated mice (Fig. 2). Differences in intensity of fluorescent-antibody staining between the saprophytic and parasitic phases have also been reported (48), but whether these differences are qualitative or quantitative is undetermined. As in the case of *H. capsulatum*, it is not known whether variations in the reaction with fluorescent antibody are related to differences in immunogenicity between the mycelial and spherule phases of *C. immitis*.

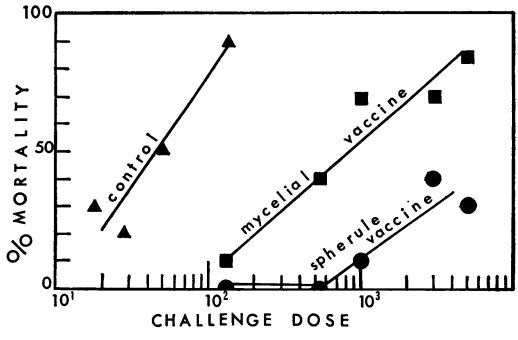

FIG. 2. *Immunogenicity of Formalin-killed coccidioidal mycelia and spherules (strain Silveira) separated after concomitant growth in a defined broth. Female mice (10 per determination) were vaccinated intramuscularly with 0.5 mg on days 0, 7, and 14, and were challenged intranasally on day 42 and observed for 33 days.*

The maturation cycle of the tissue phase, peculiar to *C. immitis*, illustrates an additional relationship between morphology and immunogenicity. Levine, Kong, and Smith (63) showed that immunogenicity increased as the spherule matured and endosporulated. The increase in immunogenicity apparently reflected concomitant biosynthetic developments in the wall, which was shown to contain virtually all the immunogens (55).

Fungal Vaccines and Assay of Potency

Inasmuch as induced immunity to fungi is influenced strongly by the morphological attributes of the vaccine preparation, as well as the routes of vaccination and challenge, interpretive comparisons of the findings of different workers are difficult. As is evident in Table 1, however, there has been general agreement that live *B. dermatitidis*, *C. immitis*, *C. neoformans*, and *H. capsulatum* are effective in animals. But agreement on the efficacy of live vaccines by different workers did not always extend to killed vaccines, possibly

TABLE 1. *Induced immunity to fungi: summary of findings on vaccination and challenge routes and morphological phase of live and killed preparations*

Organism	Live or killed	Growth phase	Host	Vaccination route	Challenge[a] route	Resistance[b]	Reference
Blastomyces dermatitidis	Live	Yeast	Guinea pigs	Intraperitoneal	Intraperitoneal	Increased	19
			Mice	Intraperitoneal	Intravenous	Not increased	110
				Intravenous	Intravenous	Increased	44
	Killed	Yeast	Mice	Intravenous	Intravenous	Not increased	44
Coccidioides immitis	Live	Spherule	Mice	Intramuscular	Intranasal	Strong	53
		Endospore	Mice	Intranasal	Intranasal	Strong	53
		Arthrospore	Mice	Intramuscular	Intraperitoneal	Strong	14
				Intranasal	Intranasal	Strong	53, 82
				Intraperitoneal	Intranasal	Strong	82
					Intraperitoneal	Strong	14
				Subcutaneous	Intranasal	Strong	82
					Intraperitoneal	Strong	14, 82
				Subcutaneous + intranasal	Intranasal	Strong	82
			Monkeys	Subcutaneous	Respiratory	Increased	15, 16, 83
			Dogs	Subcutaneous	Respiratory	Increased	12
	Killed	Spherule	Mice	Intramuscular	Intranasal	Strong	63, 64, 65
				Intraperitoneal	Intranasal	Strong	64
				Intravenous	Intranasal	Increased	64
				Footpad	Intranasal	Increased	63, 65
				Oral	Intranasal	Not increased	—[c]
				Intramuscular + intraperitoneal	Intranasal	Strong	64
				Intramuscular + intravenous	Intranasal	Increased	64
		Endospore	Mice	Intramuscular	Intranasal	Strong	62, 64, 65
				Intranasal	Intranasal	Increased	53
				Intraperitoneal	Intranasal	Strong	64
				Intravenous	Intranasal	Not increased	64
				Intramuscular + intravenous	Intranasal	Increased	64
		Spherule + endospore	Mice	Intramuscular	Intranasal	Strong	55, 61, 62
				Intranasal	Intranasal	Increased	61
				Subcutaneous	Intranasal	Strong	61, 62
				Subcutaneous + intranasal	Intranasal	Strong	61
			Monkeys	Subcutaneous + intramuscular	Respiratory	Increased	66
		Mycelial	Mice	Intramuscular	Intranasal	Increased	61
				Intranasal	Intranasal	Not increased	61

				Subcutaneous	Intranasal	Increased	61, 62
				Subcutaneous + intranasal	Intranasal	Increased	61
				Intramuscular	Intranasal	Increased	61
		Arthrospore	Mice	Intraperitoneal	Intraperitoneal	Strong	14
				Subcutaneous	Intranasal	Increased	62, 82
					Intraperitoneal	Strong	82
						Increased	29
			Monkeys	Subcutaneous	Respiratory	Increased	16
				Respiratory	Respiratory	Increased	113
Cryptococcus neoformans	Live	Yeast	Mice	Intraperitoneal	Intravenous	Increased	69, 71
				Intravenous	Intravenous	Increased	69, 71
				Footpad	Intravenous	Strong	87
	Killed	Yeast	Mice	Intraperitoneal	Intravenous	Increased	2
						Not increased	69
				Subcutaneous	Intravenous	Increased	2
						Not increased	31
				Intraperitoneal + subcutaneous	Intravenous	Increased	1, 2
Histoplasma capsulatum	Live	Yeast	Mice	Intracerebral	Intracerebral	Strong	96
				Intraperitoneal	Intracerebral	Strong	96
					Intraperitoneal	Increased	109
					Intravenous	Increased	89, 93
				Intravenous	Intracerebral	Strong	96
					Intravenous	Increased	45, 89, 92
				Subcutaneous	Intracerebral	Strong	96
	Live	Mycelial	Mice	Intratracheal	Intratracheal	Increased	25
	Killed	Yeast	Mice	Intracerebral	Intracerebral	Increased	94
				Intraperitoneal	Intracerebral	Strong	94, 95
					Intraperitoneal	Increased	109
					Intravenous	Increased	45
						Not increased	92
				Intramuscular	Intravenous	Increased	45
				Intravenous	Intracerebral	Strong	94
					Intravenous	Increased	45
						Not increased	92
				Subcutaneous	Intracerebral	Strong	94
					Intravenous	Increased	45
				Footpad	Intracerebral	Strong	94
				Oral	Intracerebral	Increased	94
					Intravenous	Not increased	45

[a] Arthrospores in all coccidioidal challenges; yeast with all other fungi except (25) where the mycelial phase of *H. capsulatum* was also used.
[b] Strong = LD_{50} displaced > eightfold; increased = LD_{50} displaced up to eightfold or to an undetermined extent.
[c] D. C. Savage, Ph.D. Thesis, Univ. of Calif., Berkeley, 1965.

because the above variables become highly critical with killed vaccines.

A very important consideration was the dose of vaccine. The extent of immunity induced by killed *H. capsulatum* (96, 109) and *C. immitis* (62, 65) was found to be dose-dependent, and, at high doses, the extent of resistance was comparable to that following sublethal infection (45, 53, 82, 96, 109). It is possible that the inefficacy of yeast phase vaccine of *B. dermatitidis* (44) was related, in part, to the dose used. The importance of dose of killed *C. neoformans* was emphasized by Abrahams and Gilleran (2), who observed that doses either above or below a certain range were suboptimal for protection. For this reason, the failure of other investigators (60, 69) to demonstrate strong resistance to *C. neoformans* in mice vaccinated with killed organisms requires further attention. However, even with live organisms, the immune response to *C. immitis* (53, 82) and *H. capsulatum* (96) was dose-dependent. Although the dependency was less pronounced than with killed organisms, its presence demonstrated that strong stimulation occurred only after a threshold of immunogens was reached. Speculations concerning the high immunogenicity of live organisms have been discussed by Salvin (99). One speculation is that the live organisms multiply, and thereby increase their immunogen content in the host. Such multiplication was evident after sublethal infection with *Cryptococcus* (69) and *Histoplasma* (95). That multiplication of attenuated *C. immitis* (53) occurred also in vivo was shown by culturing the lungs of infected mice and assaying the numbers of organisms at intervals after intranasal inoculation. Despite the use of a highly attenuated strain (LD_{50} of $>10^5$ in contrast to LD_{50} of 30 for the parent culture), *C. immitis* numbers increased from 10^2 up to 10^4 during the first 10 days (53); multiplication, however, was a transient feature, and the lungs were cleared of the fungus by 31 days after infection.

Unlike the findings on killed coccidioidal vaccines (Table 1), the growth phase was not an important consideration with live vaccines; resistance induced by 5 and 10 thousand live arthrospores was equivalent to that induced by corresponding numbers of spherules or endospores (53). With live arthrospores, multiplication of *C. immitis* in vivo is preceded by spherulation. The presence of lesions containing spherules after immunization with arthrospores or endospores has been observed by us (53), by Converse et al. (15, 18), and by Pappagianis et al. (84). It appears that the immunogenic activity of live arthrospores is associated with their morphogenesis to spherules and not with multiplication per se, as suggested by Converse et al. (15), because killed arthrospores were poorly immunogenic (15, 62, 82), whereas killed spherules and endospores were highly immunogenic (53, 62).

The production by live *C. immitis* of lesions (15, 18, 53, 84), at times distal to the vaccination site (15, 18, 84), led to a search for strains of low virulence and means to control extensive pathological reactions. Accordingly, prevaccination of monkeys with killed arthrospores was investigated and found to lessen the extent of tissue reaction after subcutaneous injection of live arthrospores. Nevertheless, a small percentage of animals still showed ulceration and regional lymphadenopathy (16). Amphotericin B treatment, concurrent with vaccination, reduced the tissue reaction of dogs to live organisms without affecting the immune response (12). However, the very requirement for antibiotic treatment with the above live coccidioidal vaccines argues against their use in man.

Although the search for coccidioidal strains of low virulence was largely successful (18, 27, 53), additional limitations in their usefulness have been uncovered recently. The highly attenuated auxotroph (27) of strain Silveira was found to be very immunogenic, but it reverted to prototrophy in vivo and regained virulence (82). Other highly attenuated cultures obtained by repeated transfers of strain Silveira in a defined broth not only gained virulence in vivo, but virulence also increased in vitro with passages on complex agar medium (53). Whether these considerations on pathogenicity and stability will apply to live vaccines prepared from the other fungi in question must await further studies.

Whereas resistance evoked by live organisms was little influenced by the parenteral route used (Table 1), the vaccination route appeared to be of major importance in fungal immunity induced by nonliving vaccines. The greatest effect was observed with *C. immitis* in mice. The intranasal route was less effective than the subcutaneous route, and the weakest immune response followed intravenous vaccination (64). Most of the intravenously vaccinated animals succumbed to a challenge dose of 5 LD_{50}, compared with >200 LD_{50} in intramuscularly immunized mice. Moreover, the lower challenge dose was lethal to most intramuscularly vaccinated mice that had also received vaccine intravenously either concurrently with, up to 49 days before, or up to 35 days after the intramuscular dose. These findings may be referable to an immunologically unresponsive state (64) produced by intravenous vaccination.

With killed *B. dermatitidis*, only the intravenous route of vaccination has been used to date, and it is ineffective (44). Whether its ineffectiveness reflects influences similar to those found with intravenous doses of *C. immitis* is unknown. The effect

of vaccination route with killed *C. neoformans* is also unknown. Although Abrahams and Gilleran (2) reported the superiority of the intraperitoneal to the subcutaneous route, the vaccinating schedules were not constant despite the use of comparable dosage, and the data therefore cannot be directly compared.

As discussed earlier, different growth phases of a fungus may vary in immunogenicity. It is not always clear whether they also vary in virulence, but the multiform characteristics of the fungi must be taken into consideration in determining the structures used in challenge studies. The capsule size of cryptococcal strains, thus far, has not been correlated with virulence (39).

With *C. immitis*, Converse (14) reported that strain M-11 increased in virulence for mice (intraperitoneally) when its arthrospores were converted to endospores in vitro. In contrast, using two other strains of high and low virulence, Pappagianis et al. (85) reported that no change in virulence occurred when the in vivo-converted endospores were isolated and used to infect mice. Since the strains and conversion procedures employed by these investigators were different, more studies are required to clarify the relationship between growth phase and virulence.

Aside from variations in the virulence of fungus strains, it is well documented that the capacity of a given strain to kill animals varies with the route of infection with the host. The LD_{50} of *C. neoformans* for mice increases when administered in the following order: intracerebrally, intravenously, intraperitoneally (26, 39, 67, 69). Similar findings in mice were reported for *H. capsulatum* (95) when all three routes were compared, and for *B. dermatitidis* (42) when the last two were studied. In monkeys, intratracheal and intravenous injections of *Histoplasma* yeast phase appeared to produce a more severe disease than that following intranasal inoculation (106). And, in dogs (72), intrathecal inoculation of *C. neoformans* had graver consequences than intravenous inoculation, but dogs were refractory to the organisms given intraperitoneally. The subcutaneous route generally produced only localized lesions with little dissemination. This was true for *C. immitis* in both mice (84) and monkeys (18). With *C. neoformans*, however, the occurrence of dissemination apparently varied with the strain used (4, 67, 87).

The intranasal route generally also caused more severe coccidioidomycosis (82) and histoplasmosis (95) than the intraperitoneal route. Although, with *C. neoformans*, Ritter and Larsh (91) reported a higher lethality by the intraperitoneal route, compared with the intranasal, our experience with this fungus (55) showed that the mortality end point following intranasal infection required a period of 4 months. It is not known whether the difference is attributable to the shorter observation period or to the fungal species. It is known, however, that immunized mice showed a lower resistance to respiratory challenge than to challenges by other routes: Grayston and Salvin (38) found that mice given killed yeast cells of *H. capsulatum* showed less severe pathological changes after intracerebral challenge than after intranasal challenge. Formalin-killed arthrospores and mycelia of *C. immitis* protected mice well against intraperitoneal, but poorly against intranasal, challenge (82). The lack of strong protection against respiratory challenge but not against intravenous challenge has also been reported for certain *Mycobacterium tuberculosis* fractions (90).

The foregoing discussion points to the importance of the challenge route in vaccine studies. The intracerebral, intravenous, and intraperitoneal routes generally allow the organisms to disseminate rapidly to various organs, whereas the respiratory route, in appropriate doses, initiates primarily a pulmonary disease (17, 35, 37, 54, 88, 91, 115) with extrapulmonary dissemination as a later occurrence. Both may be modified by immunization in the case of *C. immitis* (15, 54, 66, 117).

Time Relationships in Immunity Development

It is apparent from numerous reports on *C. immitis*, *C. neoformans*, and *H. capsulatum* that immunity induced by live organisms persists longer than that induced by killed organisms. The duration of immunity evoked by live organisms may be attributable to prolonged stimulation provided by their persistence in the host (54, 69, 95). Salvin (96) found that resistance to histoplasmosis induced by a live vaccine was first detected when the challenge was carried out 3 days after immunization and that it remained strong for at least 14 days. Immunity was also effective against *C. neoformans* (69) at 14 days, against *B. dermatitidis* (19), *H. capsulatum* (109), and *C. neoformans* (87) at 40 or 42 days, and against the latter two organisms at 90 days (45, 71). Similarly, strong immunity induced by *C. immitis* live vaccines was observed when the challenge was performed approximately 1 to 6 months later in mice (14, 53, 82) and monkeys (15, 83).

With killed vaccines, however, immunity to histoplasmosis and cryptococcosis generally declines over a relatively short period. Mice vaccinated with multiple doses of killed *C. neoformans* survived longer when challenged 7 to 14 days rather than 21 days later (2). With a histoplasmal yeast-phase vaccine, Salvin (94) reported

a decline with time in resistance to intracerebral challenge. Mice were protected against a challenge of 320 LD_{50} at 6 to 14 days postvaccination, but at 24 days they were protected only against 10 LD_{50}.

In contrast to the rapid decline in histoplasmal and cryptococcal resistance induced by killed organisms is the persistence of immunity to *C. immitis*. Strong immunity developed approximately 30 days after the first of three 0.7-mg intramuscular doses of Formalin-killed spherules (65). After 5.5 months, the mice were as well protected against a challenge of 60 LD_{50} as those challenged at 1 or 3 months (55, 57) postvaccination. However, when suppression of fungal growth in the lungs was used as an index of the immune state (54), immunity was found to decline after a 2-month postvaccination period (57). A 0.02-mg booster dose given 1 week before challenge (at >2 months postvaccination) was sufficient not only to restore the original immunity but also to augment it (57).

IMMUNOGENS IN FUNGAL STRUCTURES

Locus

The protective antigens in *C. immitis* and *H. capsulatum* have been found mainly in the cell wall. After mechanical disruption and fractionation of coccidioidal spherules, the walls and not the protoplasm contained the immunogenic activity (55). Further, the admixture of purified walls with the soluble and particulate protoplasmic fractions did not result in a preparation more immunogenic than the walls alone. Although some protective activity was demonstrable in the soluble protoplasmic fraction when it was emulsified in Freund's complete adjuvant, the activity might have been introduced from the walls during disruption of the spherules. Similarly, the yeast-phase walls of *H. capsulatum* were as immunogenic as killed whole cells, and the protoplasmic fraction contained virtually no activity (103).

In *C. neoformans* also, preliminary findings implicate the immunogenic importance of the walls. The immunogenicity of organisms was increased by enzymatic removal of their polysaccharide capsule (33). Similarly, assay of different capsular fractions, obtained by successive grindings, showed that those proximal to the wall were more immunogenic than those further removed (34). As with *C. immitis*, the activity might have been derived from the walls during the grinding. More recently, walls or their extracts were found to elicit delayed hypersensitivity reactions in guinea pigs. rabbits (5, 68, 105), and man (5), but these preparations were not tested for immunogenicity.

Although the cell wall of *B. dermatitidis* has been isolated (101), its immunogenicity is still undetermined.

Chemical Nature

The cell wall of the biphasic fungi consists of lipids, polysaccharides, proteins, and chitin-like substances (6, 7, 9, 77, 81). It is the locus of protective and serologically reactive antigens in the tissue forms of *H. capsulatum* (101) and *C. immitis* (55). It is quite resistant to mechanical disruption, in that the antigens tend to remain associated with the particulate fraction.

The spherule wall of *C. immitis*, sterilized with Formalin, is highly resistant to degradation in vivo (63, 65), and the immunogens apparently are released slowly. This is inferred from the following findings. First, the development of a strong response to vaccination required approximately 30 days (65). Second, killed spherules used for vaccination were demonstrable in intramuscular and footpad sites for at least 30 days, and in the latter site spherule wall material was detectable by radioactivity for at least 15 days and immunogens were demonstrable for at least 35 days (63). Finally, amputation of a mouse's limb above the vaccination site at 32 (intramuscular) or 40 (footpad) days postvaccination still impaired the development of immunity (63). Formalin-killed spherules (62) and their walls (*unpublished data*) are also very resistant to digestion in vitro, and appreciable amounts of soluble immunogens have not been extracted thus far by enzymatic or chemical procedures.

The high insolubility of coccidioidal immunogens in the spherule phase is a major obstacle in studies on their chemical nature. Several investigators have obtained polysaccharide-rich preparations from the mycelial phase, but their protective capacities were marginal (13, 77, 86, 113). It is possible then that protective antigens other than polysaccharide play an important role in coccidioidal immunity; recently, we have obtained indirect evidence supporting this view. As shown in Table 2, the immunogenicity of the spherules was reduced markedly by heating for 15 min at a temperature as low as 60 or 70 C. Neither the above temperatures nor the highest, 121 C, however, destroyed the immunogenicity completely. Moreover, we observed that no further reduction in immunogenicity occurred at 121 C when the exposure time was increased from 15 min to 18 hr (64). The loss in immunogenicity after autoclaving was probably qualitative, because a fivefold increase in the dose of heated vaccine did not further

TABLE 2. *Effect of heat on the immunogenicity of spherules of Coccidioides immitis strain Silveira*

Challenge dose (no. of arthrospores)	Per cent mortality[a] (30 days postchallenge)					
	Nonvaccinated control	Formalin-killed vaccine control	Vaccines heated for 15 min			
			60 C	70 C	100 C	121 C
30	40					
50	80					
100	30	10	0	10	0	0
580		10	40	30	50	70
1,400		20	40	20	60	50
2,300		0	60	90	50	50
3,400		71	40	80	66	90

[a] Seven to ten mice per determination; female mice were given 0.6 mg intramuscularly on days 0, 7, and 14, and were challenged intranasally on day 42.

enhance the resistance of mice (*unpublished data*). These findings indicate that either there are two or more immunogens or there is a single immunogen with at least two groups of determinants. One is heat-stable or, in its molecular configuration, is protected from the adverse effects of heat, and the other is heat-labile; we speculate that the former is polysaccharidal and the latter is proteinaceous.

The proteinaceous nature of the labile immunogens is further suggested by the observation that their determinant groups were protected against heat denaturation by pretreatment with Formalin. The data in Table 3 show that Formalin-killed spherules, unlike live spherules, did not lose immunogenicity after heating at 70 C. Also, several studies (13, 55, 62) have indicated that Formalin treatment rendered the spherules or mycelia highly resistant to mechanical disruption or chemical extraction and preserved the immunogenic activity for at least 20 months at 4 C (55).

In *H. capsulatum*, a protein-carbohydrate complex was isolated by Salvin and Smith (104) from culture filtrates of autolyzed yeast phase cells and was apparently as immunogenic as killed cells. In contrast, the protective capacity of a predominantly polysaccharidal preparation used by Knight et al. (51) was inferior to that of killed cells. Although both antigens were prepared by alcohol precipitation of yeast-phase material, the two findings were not necessarily contradictory; the isolation and purification procedures were not the same, nor were the vaccination and challenge regimens similar. A polysaccharidal fraction from the culture filtrate of *B. dermatitidis* yeast phase was very poorly protective (76); only a delay of the onset of disease was observed in challenged mice.

The importance of polysaccharide antigens in immunity to *C. neoformans* is indicated by the findings of Perceval (87), who used two strains of known types (22, 23) for vaccination and challenge. He found that infection with a type A conferred immunity on mice challenged with the homologous strain but not with type B. Since cross-reactions among antisera to types A, B, and C are well documented (24), further studies with additional strains are required to determine the relation of type-specific capsular polysaccharide to induced immunity. With cryptococcal fractions generally, induced immunity has been relatively weak. The polysaccharide preparations from the proximity of the wall protected animals only against low challenge doses (34, 36). Isolated cryptococcal walls are, as yet, unstudied immunogenically, and the nature and potency of the immunogens in this genus are unknown.

Specificity

The specificity of immunity induced by the fungi under consideration has been examined by cross-challenge both with heterologous organisms having antigens in common and with those not so constituted. These findings are tabulated in Table 4. Experimentation with antigenically unrelated organisms, thus far, has pointed clearly to the immunospecific nature of protection in cryptococcosis (34, 69) and coccidioidomycosis (55), but, with antigenically related organisms, the results are less certain (Table 4). Further

TABLE 3. *Effect of heat on the immunogenicity of Formalin-killed spherules of Coccidioides immitis strain Silveira*

Challenge dose (no. of arthrospores)	Per cent mortality[a] (35 days postchallenge)				
	Nonvaccinated control	Formalin-killed vaccine lot A		Formalin-killed vaccine lot B	
		Control	Heated[b]	Control	Heated[b]
10	20				
20	40				
45	50				
90	80	0	10	0	0
440		10	0	0	0
950		50	10	10	0
1,900		40	10	40	0
2,900		30	66	44	11

[a] Nine to ten mice per determination; female mice were given 0.5 (lot A) or 0.6 (lot B) mg of vaccine intramuscularly on days 0, 7, and 14, and were challenged intranasally on day 42.

[b] Spherules, killed with Formalin (0.5%), were washed three times in 0.15 M NaCl and heated at 70 C for 15 min.

TABLE 4. *Influence of pretreatment with fungal preparations on the resistance of mice to heterologous challenge*

Vaccine prepn	Challenge organisms and literature reference	
	Resistance increased	Resistance not increased
Blastomyces dermatitidis		
Yeast phase (live)	*Rickettsia typhi* (110) *H. capsulatum* (110)	*C. albicans* (96) *H. capsulatum* (96)
Yeast phase (killed)	*Mycobacterium tuberculosis* (41)	*C. albicans* (40) *H. capsulatum* (94)
Mycelial phase (killed)	*M. tuberculosis* (41)	
Candida albicans		
Yeast phase (live)		*R. typhi* (110) *H. capsulatum* (96)
Coccidioides immitis		
Spherule phase (killed)		*C. neoformans* (55) *Pseudomonas pseudomallei* (55)
Spherule phase (disrupted)	*C. albicans* (40)	
Cryptococcus neoformans		
Yeast phase (live)	*R. typhi* (110)	*Klebsiella pneumoniae* (69) *M. tuberculosis* (69) *Staphylococcus aureus* (69)
Yeast phase (killed)	*M. tuberculosis* (41)	*C. albicans* (40)
Capsular polysaccharide		*K. pneumoniae* (34) *S. aureus* (34)
Histoplasma capsulatum		
Yeast phase (live)	*B. dermatitidis* (93) *C. immitis*[a] *R. typhi* (110)	*B. dermatitidis* (96) *C. albicans* (96)
Yeast phase (killed)	*C. albicans* (40) *R. typhi* (110)	*B. dermatitidis* (94) *M. tuberculosis* (41)
Mycelial phase (killed)	*M. tuberculosis* (41)	

[a] Pappagianis and Prato, *personal communication*.

studies are therefore indicated to clarify the relationship between protective and serologically cross-reacting antigens and to rule out the possibility of nonspecific effects.

With the possible exception of one report on *C. neoformans* by Perceval (87), induced immunity was not strain-specific in experimental coccidioidomycosis (14, 15, 55, 65, 82), histoplasmosis (45, 89), blastomycosis (44), or cryptococcosis (2, 34, 69). As noted earlier, Perceval (87) compared only two strains and showed that immunity to type A did not extend to type B. Whether this will reflect type specificity and not strain specificity is unknown, since strain typing was not carried out in the studies of others (2, 69), and the relationship between type-specific capsular polysaccharide (22, 23) and immunity is undetermined.

In the above studies, both avirulent and virulent strains induced comparable immunity to the mycoses. These findings indicated that immunogenic activity was unrelated to virulence. Similarly, the capsule size of *C. neoformans* has been correlated with antigenicity (2, 49, 79) but not with virulence (39).

Of some interest in the province of specificity are findings that certain fungal preparations enhanced the resistance of mice to a variety of microorganisms. The examples listed in Table 4 emphasize the capacity of injected fungal preparations to augment innate resistance, but the nature of this nonspecific influence is little known.

MANIFESTATIONS OF THE IMMUNE RESPONSE

Survival After Challenge

The most stringent tests of efficacy in experimental vaccination are the prevention of infection after severe challenge or, failing that, the conferment on the host of resistance sufficient to clear infecting organisms rapidly without the development of serious illness. These criteria, seldom approached with bacterial and viral vaccines, have not been met by fungal vaccines. However, important facets of them have been achieved. Live cryptococcal (69, 87), histoplasmal (96), and coccidioidal (15, 53, 82) preparations have enabled animals to withstand severe challenge and to survive for extended periods. Equally striking has been the efficacy of killed coccidioidal (53, 55,

TABLE 5. *Effect of vaccination on multiplication of Coccidioides immitis strain Silveira in the organs of mice challenged with seven arthrospores*[a]

Day after challenge	Avg[b] no.[c] of recovered viable fungal units per organ					
	Lungs		Liver		Spleen	
	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated
0	5	2	0	0	0	0
3	<1	<1	0	0	0	0
5	230	630	0	0	0	0
7	19,000	890	<1	0	0	0
10	15,000	200	0	0	0	0
13	63,000	160	120	0	2	0
18	43,000	2,200	73	0	40	0
33	220,000	32,000	43	110	>670	45
91	130,000	2,800	270	<1	140	0
91	13/14[d]	5/11	6/14	0/11	5/14	0/11

[a] Results from reference 54. Female mice were given 0.8 mg of Formalin-killed spherules intramuscularly on days 0, 7, 14, and 21 and were challenged intranasally on day 50.

[b] Eight to nine mice per determination.

[c] Numbers from surviving control animals only (approximately 90%); there were <2% deaths in the vaccinated group.

[d] Number of survivors with lesions.

57, 65) and histoplasmal (94, 95) vaccines. Vaccinated mice survived challenge with >200 LD_{50} for long periods and, with low doses of *C. immitis* (54), showed progressive reduction in fungal numbers from day 32 to at least 8 months postinfection. With a booster dose, as will be described, a majority of the mice cleared their tissues completely of *Coccidioides* (57).

Inhibition of Fungal Multiplication and Dissemination

The suppression of coccidioidal multiplication in intranasally challenged mice vaccinated previously with killed spherules or their walls approached 500-fold (54). As shown in Table 5, restricted multiplication in the lungs was associated with reduced dissemination to the liver and spleen; extrapulmonary lesions, present in 36% of the control animals, were not seen in any of the immunized mice.

Suppressed histoplasmal growth has also been reported in mice immunized with live or killed organisms and challenged intranasally, intraperitoneally, intravenously, or intracerebrally, and in guinea pigs challenged intraperitoneally (95, 96). Initially after challenge, multiplication was most pronounced at the site of infection in both control and immunized mice, and the extent of brain and kidney involvement varied somewhat with the route of infection (95). However, unlike *C. immitis*, histoplasmal growth occurs largely within cells of the reticuloendothelial system, and the organisms or lesions containing them have been found invariably in the liver and spleen regardless of the challenge route (37, 38, 59, 88, 92, 95, 96, 101). Rowley (92) indicated that inhibited growth was demonstrable in intravenously challenged mice only by the use of live vaccines. However, the 6-day postchallenge interval employed by Rowley might have been too short to detect low levels of inhibition; in studies with *H. capsulatum* (95), *C. immitis* (54), *M. tuberculosis* (54), and *C. neoformans* (2, 69), this aspect of immunity was observed only in relatively advanced infections.

The severity of blastomycosis was modified by prior vaccination (19, 44), but no data on fungal growth were presented. With *C. neoformans*, the lungs and then the brain were primarily involved after intranasal infection (35, 91), but the influence of vaccination with live (69, 71) or killed (2) organisms on fungal growth has been studied only in intravenously challenged mice. Cryptococcal growth in vaccinated animals was suppressed in the brain (2, 69, 71) and kidneys (69) within 7 days postchallenge. Growth inhibition in the liver and spleen, the organs showing the least involvement, was marked (2, 69), and there was also inhibition of growth in the lungs (69).

Notwithstanding the suppressed multiplication in immunized animals, *Cryptococcus* persisted at least for 3 months (69), *Histoplasma* for 4.5 months (95), and *Coccidioides* for 8 months (54).

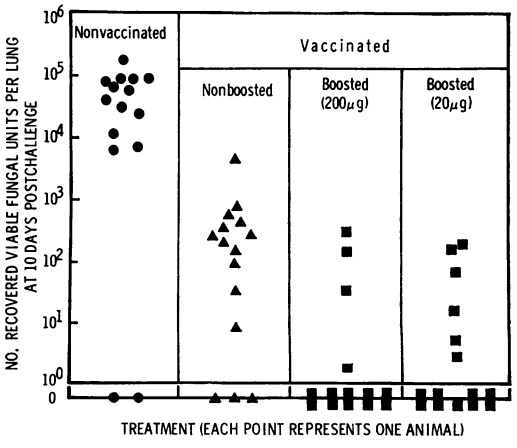

FIG. 3. *Effect of a booster dose of spherules at 7 days before challenge on Coccidioides immitis numbers in mouse lungs. Mice were given intramuscularly 0.5 mg of Formalin-killed spherules on days 0, 7, 14, and 21, boosted on day 106, and challenged intranasally with 29 arthrospores on day 113 (57).*

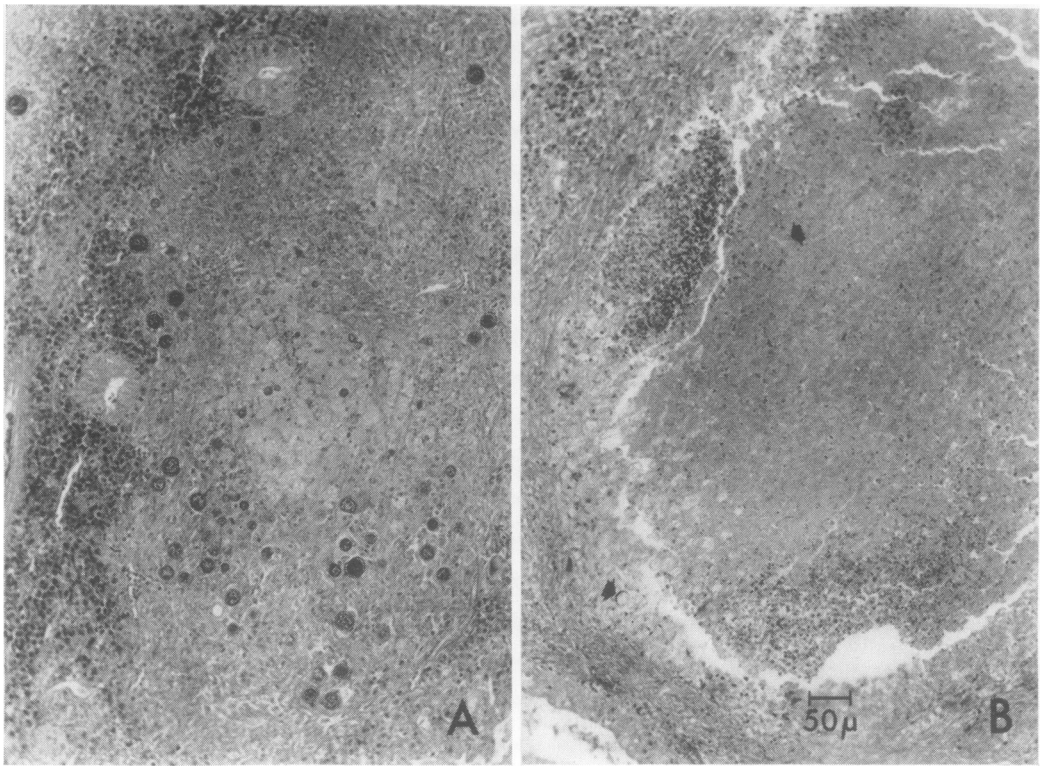

FIG. 4. *Pulmonary lesions in mice at 56 days after intranasal infection with 26 arthrospores of Coccidioides immitis (strain Silveira). (A) Control, showing numerous spherules and poor epithelioid response. (B) Vaccinated (Formalin-killed spherules), showing a well circumscribed necrotic area with few spherules (indicated by arrows).*

Clearance of fungus from the primary organs of involvement was observed in some animals challenged with *C. neoformans* at 6 months (2) and in some animals challenged with low doses of *C. immitis* at 8 months (54). Although coccidioidal immunity, as determined by growth inhibition, waned by the 2nd month after vaccination, a booster dose at this time not only restored but also enhanced it. As illustrated in Fig. 3, coccidioidal multiplication was suppressed up to 10^5-fold in the lungs of boosted mice, and >60% of them were no longer infected by the 10th day postchallenge. The booster response in *Coccidioides*-vaccinated mice was most pronounced when the booster was given at 6 or 7 days before challenge (57), and it was not demonstrable with a heterologous organism, *C. neoformans*.

Enhanced Cellular Response

An inflammatory cell response characterizes pulmonary coccidioidal disease. Its close association with resistance is strongly implied by observations that it is enhanced by vaccination and augmented by a booster dose of vaccine (57). The cellular infiltration began within 24 hr after challenge in immunized animals, and total cell numbers recovered in tracheobronchial washes reached a maximum at approximately 3 days in boosted and 6 days in nonboosted mice, compared with approximately 8 days in nonvaccinated mice (57). This early rise in inflammatory cell numbers in the lungs of vaccinated animals correlated with early clearance activity in this organ (54, 57). The cell types consisted of polymorphonuclear neutrophils, lymphocytes, macrophages, and eosinophils, but the relative importance of each in coccidioidal immunity is unknown. Both spherules and arthrospores have been found in the macrophages within the first two days after infection (D. C. Savage, Ph.D. Thesis, Univ. of Calif., Berkeley, 1965).

At the 10th day postchallenge, the total numbers in the vaccinated groups had declined (57), and this was associated with early granulomatous formations in the lungs (54). As shown in Fig. 4, the resultant granulomatous lesions were well circumscribed and contained very few spherules. The contrasting necrotic lesions in the lungs of control survivors contained many spherules and were poorly organized. These histopathological

differences were in accord with the data on fungal numbers, and appeared to account for dissemination in the nonimmunized mice (Table 5; 54).

Similarly, roentgenological and histological studies in vaccinated monkeys (15, 66, 83) and guinea pigs (117) challenged by the respiratory route showed distinctly less pulmonary and extrapulmonary involvement than in control animals. And, in intraperitoneally challenged mice, live (14) or even killed arthrospores of low immunogenicity (13, 14) conferred a capacity to localize the infection and limit the dissemination. This was also the case with *B. dermatitidis* in previously infected guinea pigs (19) and mice (44), and with *H. capsulatum* in mice vaccinated with live (38, 89, 96, 108) or killed yeast cells (38, 95, 108).

In the study with *H. capsulatum* by Grayston and Salvin (38), brain involvement in control mice was extensive after intracerebral challenge, but there was only minimal inflammatory infiltrate in mice vaccinated with live or killed organisms. Differences in the liver were less marked, but, here too, granulomas were few and well organized in immunized mice. The spleens in both groups contained many organisms but few lesions. The killed vaccine, however, appeared to be less effective against intranasal challenge than against intracerebral challenge. But, in this study, pathological involvement after intranasal challenge was limited, and lung and liver lesions in the control group were resolved spontaneously.

Reduced tissue involvement was also observed in vaccinated mice challenged with *C. neoformans*, but the importance of inflammatory cells in cryptococcal immunity is less certain. The predilection of *C. neoformans* for the brain has been well established, and vaccination suppressed fungal proliferation in that organ (2, 69, 71). However, whereas one study reported no evidence of cellular infiltration in the brain of either control or immunized mice after challenge (2), another reported an intensive cellular response in immunized animals (71).

Delayed Hypersensitivity

Delayed dermal hypersensitivity reactions to the four fungi have been elicited in infected guinea pigs (5, 21, 28, 47, 52, 58, 74, 76, 105, 111), rabbits (21, 75), monkeys (53, 106, 112), dogs (112), and rats (80). Killed organisms or their fractions also were effective in sensitizing guinea pigs (58, 68, 97, 111, 117, 118), rabbits (68, 86, 118), and monkeys (66, 113).

Because of the relative difficulty in skin testing in the mouse, delayed hypersensitivity in this species was tested systemically or in the footpad. Early deaths in mice previously vaccinated with live or killed *H. capsulatum* were produced by intravenous injection of organisms (8) or intraperitoneal injection of organisms in mucin (97, 98). This state of hypersensitivity persisted for 14 months (8). With *C. neoformans* (87), the footpad of previously infected mice showed accelerated "swelling" after an injection of live or killed organisms, and it took many days to subside. Whether these events represent true delayed hypersensitivity reactions is unknown. With *C. immitis*, both infected mice and mice vaccinated with killed spherules developed a state of delayed hypersensitivity which persisted for many months and was characterized by a cellular infiltrate comprised of $>90\%$ of mononuclear cells at 72 hr (56). Of importance is the finding that the mouse required >100 times the concentration of coccidioidin generally used to elicit a delayed reaction in man or guinea pigs. This may explain in part the difficulties encountered by many in studies on delayed hypersensitivity reactions in mice.

MECHANISMS OF THE IMMUNE RESPONSE

In general, the mechanisms of immunity and its development have been little studied in the mycoses. The present section of this report, therefore, is concerned with work which for the most part pertains tangentially to immune mechanisms. In this category are the effects on resistance of adjuvants, nonspecific treatments, and serum and cellular factors.

Effects of Adjuvants and Other Preparations

Gadebusch (31) observed that resin-coupled capsular polysaccharide of *C. neoformans* was released more slowly than unbound polysaccharide from subcutaneous inoculation sites in mice, with the result that survival time after challenge was extended. The administration of *Bordetella pertusis* in conjunction with cryptococcal and coccidioidal vaccines also increased survival in mice (1, 13); the effect was not referable to a detectable antibody response in the case of *Cryptococcus* (1). In contrast, the incorporation of Freund's adjuvants with killed cells of *C. immitis* (Table 6) or *C. neoformans* (1) suppressed immunity development. There was some potentiating effect, however, when Freund's complete adjuvant was incorporated with a soluble coccidioidal fraction (55). These data and others (63, 64) led to the speculation that the immune response to coccidioidal vaccines depended critically on the rate of immunogen release from vaccination depots.

Bacterial endotoxin, given 7 days before challenge with *C. neoformans*, afforded protection to mice (71); fungal growth was suppressed (69, 71).

TABLE 6. *Effect of Freund's adjuvant on immunogenicity of Formalin-killed spherules of Coccidioides immitis strain Silveira*[a]

Challenge dose (no. of arthrospores)	Per cent mortality[a] (29 days postchallenge)					
	Saline			Spherules (1.6 mg)		
	None	In Freund's incomplete adjuvant	In Freund's complete adjuvant	Alone	In Freund's incomplete adjuvant	In Freund's complete adjuvant
15	10	30	10			
30	70	50	50			
60	90	50	40			
160	50	90	90	0	0	20
670				20	60	50
1,500				50	80	50
4,300				20	90	70

[a] Ten mice per determination. Intramuscular doses on days 0 and 14 to female mice; intranasal challenge on day 49.

After infection, both enhanced cellular and agglutinin responses were observed, and the latter was correlated with the suppression of fungal multiplication (71). However, growth inhibition was also noted in specifically immunized mice that showed primarily an enhanced cellular response with very low or no antibody titers.

Humoral and Cellular Factors

Circulating antibodies. Precipitating and complement-fixing antibodies occur in coccidioidomycosis of dogs (112), rabbits (10, 116), and monkeys (17, 66, 112), but, as is the case in man, their role in immunity is not known (66, 112). The recognition of antibodies potentially associated with resistance is complicated by the consideration that coccidioidin, the most widely used antigen, is a complex preparation and itself is virtually nonimmunogenic (86). Purified immunogens are not yet available.

Preliminary evidence that this aspect of immunity might be resolved with suitable antigens is the observation that spherule-vaccinated and boosted mice developed precipitins demonstrable with a soluble spherule fraction (55) but not with coccidioidin (57). However, the role of these antibodies in resistance is still obscure, because they conferred no protection on recipient mice. Similarly, sera from homologous and heterologous species containing antibodies to *C. neoformans* (1, 32, 69, 70, 71) and *H. capsulatum* (92, 99) were ineffective in passive protection trials.

However, in none of the foregoing studies on passively transferred resistance with serum were isologous mice employed. This limitation in conjunction with the chronic nature of fungal diseases could have masked the effects of humoral factors in resistance.

Cellular response. In contrast to the failure in transferring immunity to *C. immitis* passively with serum was the moderate success obtained with transferred splenic cells in mice (57). The recipient animals showed an augmented capacity to restrict the multiplication of infecting organisms in their lungs, but unequivocal conclusions were not possible because cells from nonimmunized donors or the suspending menstruum for the cells potentiated fungal growth after intranasal challenge. The importance of a cellular role in immunity, however, was indicated by yet other findings. The infiltration of inflammatory cells in *Coccidioides*-vaccinated mice in response to challenge was more rapid than in nonvaccinated mice (57). With *C. immitis* (15, 54, 66, 83, 117) as well as *H. capsulatum* (38), such an accelerated infiltration was associated with the development of granulomatous lesions that were fewer and better circumscribed than those in control mice; with *C. neoformans*, inflammatory responses following nonspecific or specific treatments were associated with enhanced resistance (71).

Phagocytosis. The role of phagocytosis in immunity in general is still a subject of much study and discussion, and the few observations on in vitro phagocytosis in the fungi do not establish its function in resistance. With *H. capsulatum*, an intracellular organism, contradictory results were reported. In one study, the extent of fungal growth in macrophages from vaccinated mice was the same as that in macrophages from control mice (46), but, in others, prior vaccination increased the digestive capacity of macrophages (78, 119). Coccidioidal arthrospores and spherules were found in lung macrophages during the first 2 days after infection of both nonvaccinated and vaccinated mice. Subsequently, the numbers of ingested and extracellular organisms declined more rapidly in the vaccinated than in the control group (57; D. C. Savage, Ph.D. Thesis, Univ. of Calif., Berkeley, 1965), but actual intracellular digestion of spherules by phagocytes was not ascertained.

That phagocytosis may play a role in cryptococcal immunity is suggested indirectly by the observations that organisms with small capsules were more easily phagocytized than those with large capsules (11, 32), and that small capsule strains were more antigenic (49, 79) and protective (2) than large capsule strains.

Delayed hypersensitivity. Although the delayed reaction in man is a reliable index of previous

exposure to the biphasic fungi, and possibly also to *Cryptococcus*, its relationship to fungal immunity, as in microbial diseases, remains unresolved. We have found that about one-half of the *Coccidioides*-infected survivors and one-third of the spherule-vaccinated mice do not exhibit a footpad reaction (56) but are nevertheless immune. Similar findings recently were reported in guinea pigs (73).

CONCLUDING REMARKS

The findings on the fungi reviewed above present an emerging picture of orderly immunological relationships: experimentally induced immunity against them is highly effective, as it is against a variety of microorganisms; the determinants of immunogenicity, the locus of immunogens, the time relationships in immunity development, and the manifestations of resistance are fundamentally in accord with those described for bacteria; related immunological phenomena—delayed hypersensitivity, unresponsiveness, specificity, and cross-reactivity—appear to parallel corresponding phenomena described for bacteria and viruses; and the complex relationships of cells and antibodies in defense have features in common with selected bacteria and viruses.

Yet, fungal immunology has received less attention than viral and bacterial immunology. We have referred to possible reasons for this in the introductory remarks. But, as epidemiological and ecological knowledge expands, public health statistics point increasingly to the desirability of fungal vaccines, especially in selected regions of edemicity. This consideration, and the more basic one of how hosts cope with organisms often larger than their phagocytes, denote, in our judgment, topics for rewarding study.

ACKNOWLEDGMENTS

This investigation was supported by the Office of Naval Research and the Bureau of Medicine and Surgery, United States Navy, under a contract between the Office of Naval Research and the Regents of The University of California.

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