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Delayed Dermal Hypersensitivity in Mice to Spherule and Mycelial Extracts of *Coccidioides immitis*

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ABSTRACT

KONG, YI-CHI M. (University of California, Berkeley), D. C. SAVAGE, AND LEIGHTON N. L. KONG. Delayed dermal hypersensitivity in mice to spherule and mycelial extracts of *Coccidioides immitis*. J. Bacteriol. 91:876–883. 1966.—A delayed hypersensitivity reaction to spherule and mycelial extracts of *Coccidioides immitis* was elicited in the footpads of mice vaccinated with killed spherules. Emulsification of the spherules with Freund's adjuvants was unnecessary, but a high concentration of antigen was required to elicit the reaction. Injection of the extracts produced, initially, a swelling which subsided within 4 hr, and then induration, which began at 6 to 8 hr and reached a maximum at 24 hr. The time course of the reaction corresponded to that of the tuberculin reaction in BCG-vaccinated mice. The histological response to coccidioidal extracts was characterized by the early infiltration of both polymorphonuclear and mononuclear cells, and the subsequent predominance of mononuclear cells at 24 to 48 hr. By 72 hr, the mononuclear cells comprised >90% of the cellular infiltrate. Animals infected intranasally with arthrospores (1 to 5 LD_{50}) reacted negatively before and during the crisis period; thereafter (by 28 to 31 days after infection), up to 50% of the survivors showed a delayed reaction.

The presence of delayed dermal hypersensitivity to coccidioidin without sensitivity to cross-reacting antigens is strong evidence of previous exposure to *Coccidioides immitis* (19). The delayed reaction has been demonstrated in man and in a variety of animals, but has not been reported in mice. This study shows that mice developed delayed reactivity in the footpad after vaccination with killed spherules (12, 13) or infection with arthrospores. The nature of the reaction was determined both macroscopically and microscopically, and was observed to be similar to tuberculin hypersensitivity.

MATERIALS AND METHODS

Sensitization. Formalin-killed spherule vaccines from *C. immitis* strain Silveira were prepared and purified as described previously (14). A total dose of 2.0 to 2.4 mg was given intramuscularly in 3 or 4 weekly injections (0.1 ml) to female albino mice of the Namru strain (6), beginning at 5 to 7 weeks of age (18 to 21 g). In one case, pulverized spherules (Savage, Ph.D. Thesis, Univ. California, Berkeley, 1965) were employed. In some experiments, Freund's complete and incomplete adjuvants (Difco) were incorporated into the spherule suspensions at 1:1 ratios, and the animals received 1.0 to 2.0 mg of emulsified spherules in two intramuscular or subcutaneous injections (0.1 ml) separated by 1 week. Other animals were immunized by the above routes with 1.0 mg of phenol-killed BCG (kindly supplied by D. W. Weiss of the Dept. of Bacteriology) in either adjuvant. Also employed were mice infected with 15 to 130 (1 to 5 LD_{50}) arthrospores by the intranasal route (18).

Antigenic preparations. Dilutions of 1:10 to 1:500 of old tuberculin (OT; Eli Lilly & Co., Indianapolis, Ind). and 5 μg of purified protein derivative (PPD; Parke, Davis & Co., Detroit, Mich.) in 0.15 M NaCl (saline), prepared just before injection, were used for testing BCG-vaccinated mice. Spherule-vaccinated mice were tested with one or more of the following four coccidioidal preparations. (i) A filtrate (Millipore HA, 0.45 μ) of a spherule culture having <1% hyphae by weight, grown in modified Converse medium (13) supplemented with 1% Casamino Acids (Difco) and stored at 4 C for 9 months, was diluted 1:10 with saline and designated "spherulin." (ii) Coccidioidin, Lot 64-D3, was concentrated threefold in vacuo over P_2O_5 and was designated concentrated coccidioidin. (iii) Coccidioidin, Lot 66–71, was dialyzed against saline containing 0.01% Merthiolate at 4 C for 3 days, and was designated dialyzed coccidioidin (both lots were mycelial extracts, kindly supplied by C. E. Smith of the School of Public Health). (iv) A polysaccharide

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fraction of coccidioidin, obtained from Marcus (Marcus, Aoki, and Hill, Federation Proc. **24**:182, 1965) was dissolved in Merthiolated saline and tested at a dose of 0.1, 1.0, 10, or 50 μg. Nonvaccinated mice were also given the above preparations; the effect of diluent was controlled by injecting 0.15 or 0.45 M NaCl, with or without Merthiolate, to contralateral footpads of both nonvaccinated and vaccinated mice.

Footpad testing. All injections were made with a 27- or 30-gauge needle into a hind footpad. OT and PPD were given in a volume of 0.05 ml at 3 weeks after vaccination with BCG. For spherule-vaccinated or arthrospore-infected mice, the volume of fungal extracts was later reduced from 0.05 to 0.03 ml. Infected mice were tested at 15, 22, 28, and 31 days after intranasal exposure. Vaccinated mice were tested at 3 weeks to 6.5 months after vaccination; the interval is not specified in each instance in the text because no significant variation in the percentage of reactors with time was observed. Footpad reactions were recorded at 30 min, and 1, 2, 4, 6 (or 8), 24, and 48 hr after antigen injection, and were graded as negative, $\pm$ (erythema only), 1+ (moderate induration), 2+, or 3+, depending on the intensity of the induration.

Histological studies. The hind feet of 10 nonvaccinated and 22 spherule-vaccinated mice were depilated with Nair (Carter Products, Inc., New York) 48 hr before testing. At the time of testing, no erythema was visible, and each animal received 0.03 ml of dialyzed coccidioidin in the right footpad and Merthiolated saline in the left footpad. At 1, 6, 24, 48, or 72 hr after injection, two to six animals in each group were killed by cervical fracture. Their hind feet were amputated and fixed in Technicon FU-48. The digits were then removed and the remainder was decalcified for 3 hr with Decal (Omega Chemical Co., Garden City Park, N.Y.), embedded in paraffin, sectioned, and stained with hematoxylin-eosin.

RESULTS

Delayed hypersensitivity reaction in BCG-vaccinated mice. Namru mice were tested for their capacity to exhibit a delayed hypersensitivity reaction to the tuberculins OT and PPD. Although injection of either preparation or the saline control into the footpads of control and vaccinated mice produced moderate swelling and erythema lasting up to 4 hr, only OT elicited a delayed reaction (1+ or 2+) in the vaccinated mice. The reaction was visible from 6 to 48 hr, with maximal induration at 18 to 24 hr (Fig. 1) after injection. Incorporation of Freund's adjuvants with the BCG vaccine increased the number of positive reactors from 2 of 20 (BCG only) to 10 of 19 (incomplete adjuvant) and 12 of 20 (complete adjuvant). In addition, the route of vaccination appeared important in tuberculin hypersensitivity; the subcutaneous route produced >50% positive reactors, but intramuscular vaccination sensitized only 20% of the animals, despite the use of a complete adjuvant.

Delayed hypersensitivity reaction in spherule-vaccinated mice. Having established that a tuberculin reaction was inducible in Namru mice, the footpad reactions to coccidioidal extracts of mice vaccinated with spherules alone or spherules incorporated with Freund's adjuvants were investigated. Unlike BCG in adjuvant, subcutaneous injection of spherules in adjuvant often resulted in sloughing off of the nodule, and the intramuscular route was employed subsequently. As in footpads injected with OT or PPD, initial inflammation was elicited by spherulin in both control and vaccinated mice. However, a delayed reaction was observed only in the footpads of vaccinated mice; it lasted from 6 to 48 hr and was maximal at approximately 24 hr after injection. Figure 2 shows that the degree of delayed induration at 24 hr was similar in the footpads of vaccinated mice, whether or not adjuvant was used. Also,

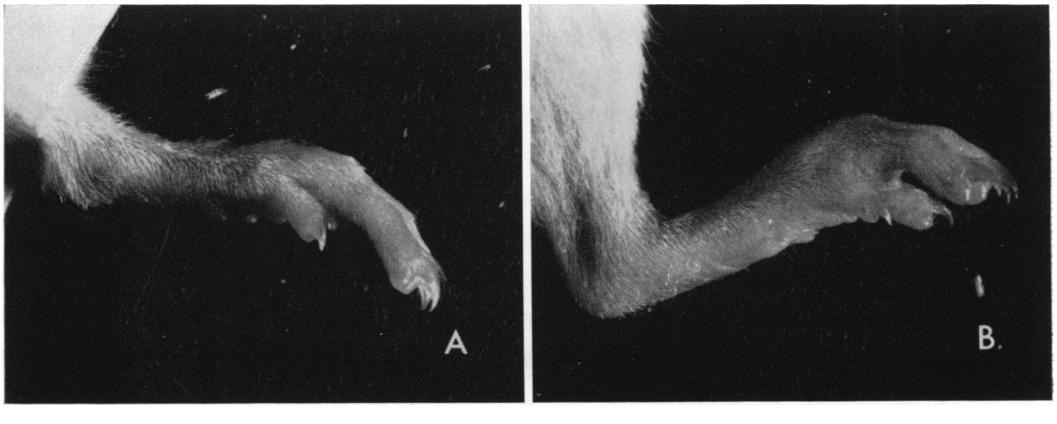

FIG. 1. *Footpad reaction of BCG-vaccinated mice to OT at 24 hr. (A) Control; (B) vaccinated, showing induration.*

the numbers of positive (1+ to 3+) reactors in the three groups were essentially the same, and were 16 of 19, 14 of 19, and 15 of 20 in groups receiving, respectively, spherules-complete adjuvant, spherules-incomplete adjuvant, and spherules alone. Interestingly, the spherule vaccine, in contrast to BCG, was a good sensitizing agent, even in the absence of adjuvant.

Spherulin proved to be very toxic, however, and regularly elicited an immediate 2+ or 3+ reaction in the footpads lasting for 4 hr. Therefore, we tested coccidioidin, lot 64-D3, which elicited a delayed reaction at a 1:100 dilution in man (C. E. Smith, *personal communication*) and monkeys (15). Although early inflammation was less persistent with the 1:100 dilution (as with saline) than with spherulin, no delayed reaction was observed in vaccinated mice. When used undiluted, this coccidioidin elicited a delayed reaction in 10% of the animals. And, only when it was concentrated threefold did approximately 75% of the vaccinated animals respond at 24 hr (Fig. 3). However, concentrated coccidioidin was also highly irritating; 100% of both control and vaccinated mice showed a 2+ or 3+ swelling at 1 hr after injection (Fig. 3).

The possibility that a purified polysaccharide fraction might contain less toxic material than spherulin or concentrated coccidioidin was also studied. At doses of 0.1 to 50 μg, the polysaccharide fraction elicited, in both control and vaccinated mice, an early inflammatory response which subsided at 2 hr. However, only 10% of the vaccinated mice responded with a delayed reaction to the 0.1- and 1.0-μg doses and 55% to the 10-μg dose. After injection of 50 μg of polysaccharide into the foodpad, induration was observed in all the immunized animals at 24 hr. However, since this amount also elicited a strong immediate reaction, which persisted for at least 4 hr longer than that in control mice, the actual number of animals showing a delayed reaction could not be ascertained.

Delayed hypersensitivity reaction in infected mice. Most animals infected intranasally with arthrospores at 1 to 5 LD_{50} were acutely ill by days 15 and 22. They showed an early inflammatory response, but exhibited no delayed reaction after testing with either spherulin or concentrated coccidioidin. By 28 and 31 days, however, the

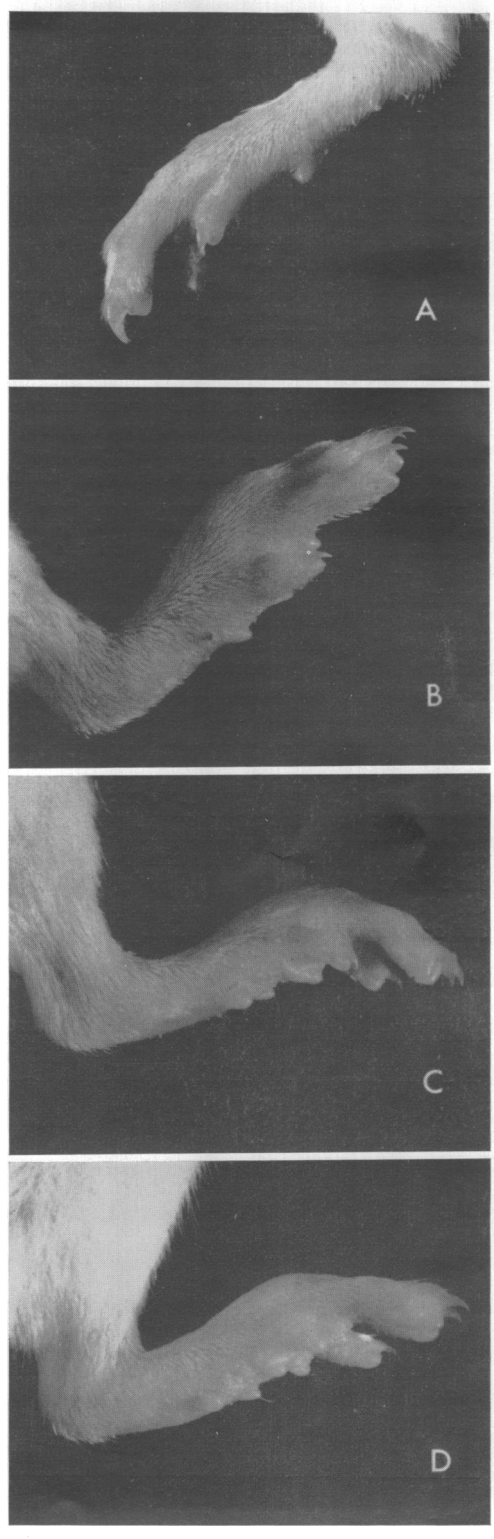

FIG. 2 *Effect of incorporating Freund's adjuvants with spherule vaccines of Coccidioides immitis on footpad reactions of mice to spherulin at 24 hr. (A and B) Vaccine in Freund's complete adjuvant: (A) left footpad, saline; (B) right footpad, spherulin. (C and D) Tested with spherulin: (C) vaccine in Freund's incomplete adjuvant; (D) vaccine alone.*

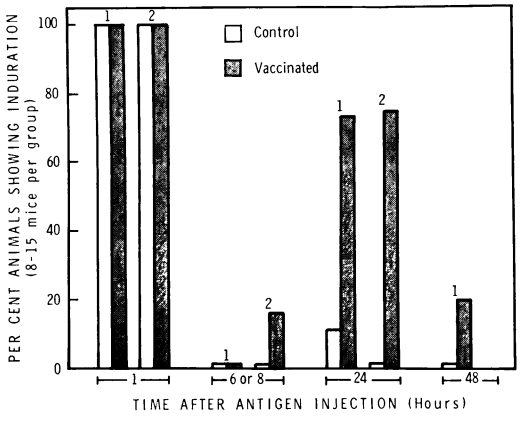

FIG. 3. *Changes in footpad reactions of nonvaccinated and spherule-vaccinated mice within 48 hr after injection of concentrated coccidioidin. Expt. 1, vaccinated with pulverized spherules and tested at 5 months after vaccination. Expt. 2, tested at 6 months after vaccination (no 40-hr data).*

crisis period had passed, and up to 50% of the survivors showed a delayed reaction.

Cellular response in the footpads of spherule-vaccinated mice. Dialyzed coccidioidin contained less irritating material than spherulin or concentrated coccidioidin, and, at high concentrations, elicited a delayed reaction in 90 to 100% of the immunized mice. It was used, therefore, for histological studies. Although initial swelling was still observed in the footpads of control mice and mice immunized 6.5 months previously, all 16 immunized mice showed a delayed reaction at 24 hr, and some also at 6 and 48 hr after injection.

Microscopically, the footpads of control mice given Merthiolated saline showed a minimal inflammatory infiltration at 1 hr and a mild cellular reaction at 6 and 24 hr. The infiltrate consisted primarily of polymorphonuclear cells, including some eosinophils, and some mononuclear cells at 6 hr and a few more mononuclear cells at 24 hr. By 48 and 72 hr, the footpads had almost returned to normal, with a few scattered polymorphonuclear and mononuclear cells remaining. The inflammatory response in the footpads of control mice given dialyzed coccidioidin was also slight, but was somewhat more intensive than the reaction in the contralateral footpads given saline. Many polymorphonuclear and some mononuclear cells were present in subcutaneous tissues at 1 hr after injection, and a few infiltrated the dermis at 6 hr. The nonspecific response was moderate at 24 hr, with a little edema and either polymorphonuclear or mononuclear cells in the majority, and, by 48 hr, the number of polymorphonuclear cells had decreased. By 72 hr, the cellular response had become minimal; some mononuclear cells were observed in and around the capillaries of deep subcutaneous tissues.

Cellular reactions to coccidioidin in the foot pads of vaccinated mice differed from those described above for control mice. These differences were not great initially; at 1 hr, the inflammatory infiltrate, minimal in the footpad given saline (Fig. 4A), was also slight in the contralateral footpad given coccidioidin (Fig. 4B). The coccidioidin elicited a little vascular congestion and the infiltration of some polymorphonuclear cells in the subcutaneous tissues with a few in the dermis. However, by 6 hr, the inflammatory response in the coccidioidin-injected pad had increased somewhat (Fig. 4D); there were some congested blood vessels, but negligible edema and moderate perivascular infiltration of predominantly polymorphonuclear cells. In contrast, cellular infiltrate in the saline-injected pad was slight at 6 hr (Fig. 4C).

Most vaccinated animals exhibited a 2+ or 3+ induration in the footpads given coccidioidin at 24 hr, and the histological sections (Fig. 5A) showed an acute, generalized inflammatory response from the sarcolemmal sheath to the epidermis. The cells (Fig. 5B) were predominantly polymorphonuclear in type, with some eosinophils, but there were also many mononuclear cells. Some congestion was observed in the adipose tissue, but edema was minimal and hemorrhage occasional. The footpad of one animal showed only a 1+ induration at 24 hr, and the sections had many more mononuclear and fewer polymorphonuclear cells than those described above. By 48 hr, the cellular reaction was still intensive and generalized, but many more mononuclear than polymorphonuclear cells were observed, and the ratio was approximately 2:1. At 72 hr, whereas the control footpads showed negligible or minimal cellular response, the cellular infiltrate in the coccidioidin-injected footpads of vaccinated mice was still extensive and consisted of >90% mononuclear cells (Fig. 5, C and D), many of which were located around small blood vessels and nerves. During the 72-hr period, neither necrosis nor cells of the plasma cell series were observed.

DISCUSSION

Delayed hypersensitivity reactions in mice have not been elicited regularly by skin or footpad tests (2) until recently (4, 16; Paas and Friedman, Bacteriol. Proc., p. 55, 1965; Rowlands, Crowle, and Russe, Federation Proc. **23**:286, 1964). The variation in results may be related,

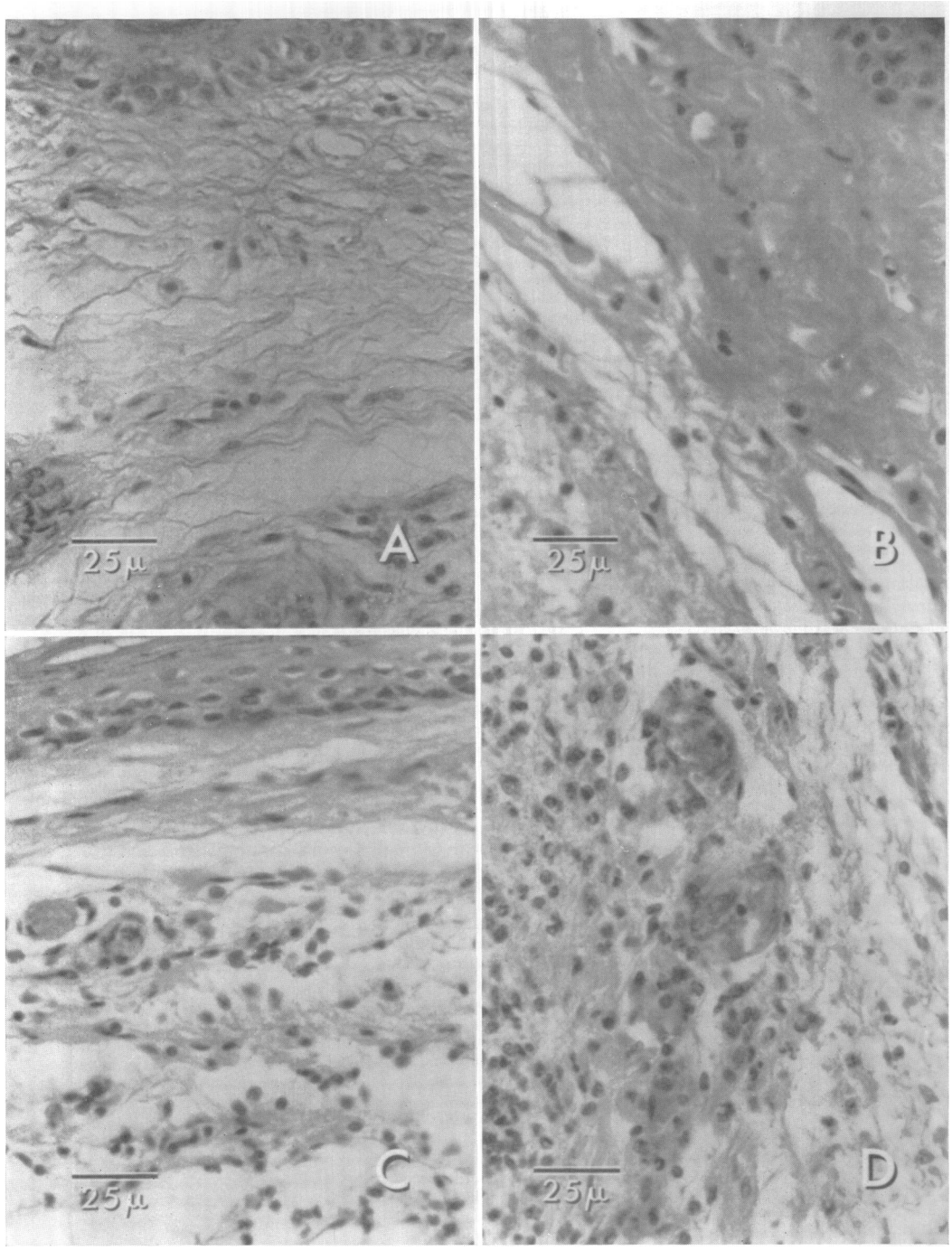

FIG. 4. *Cellular response in the footpads of spherule-vaccinated mice at 1 and 6 hr after injection of saline or dialyzed coccidioidin. At 1 hr: (A) saline; (B) coccidioidin, both showing minimal cellular response. At 6 hr: (C) saline, weak cellular response; (D) coccidioidin, moderate cellular infiltration, polymorphonuclear cells predominant.*

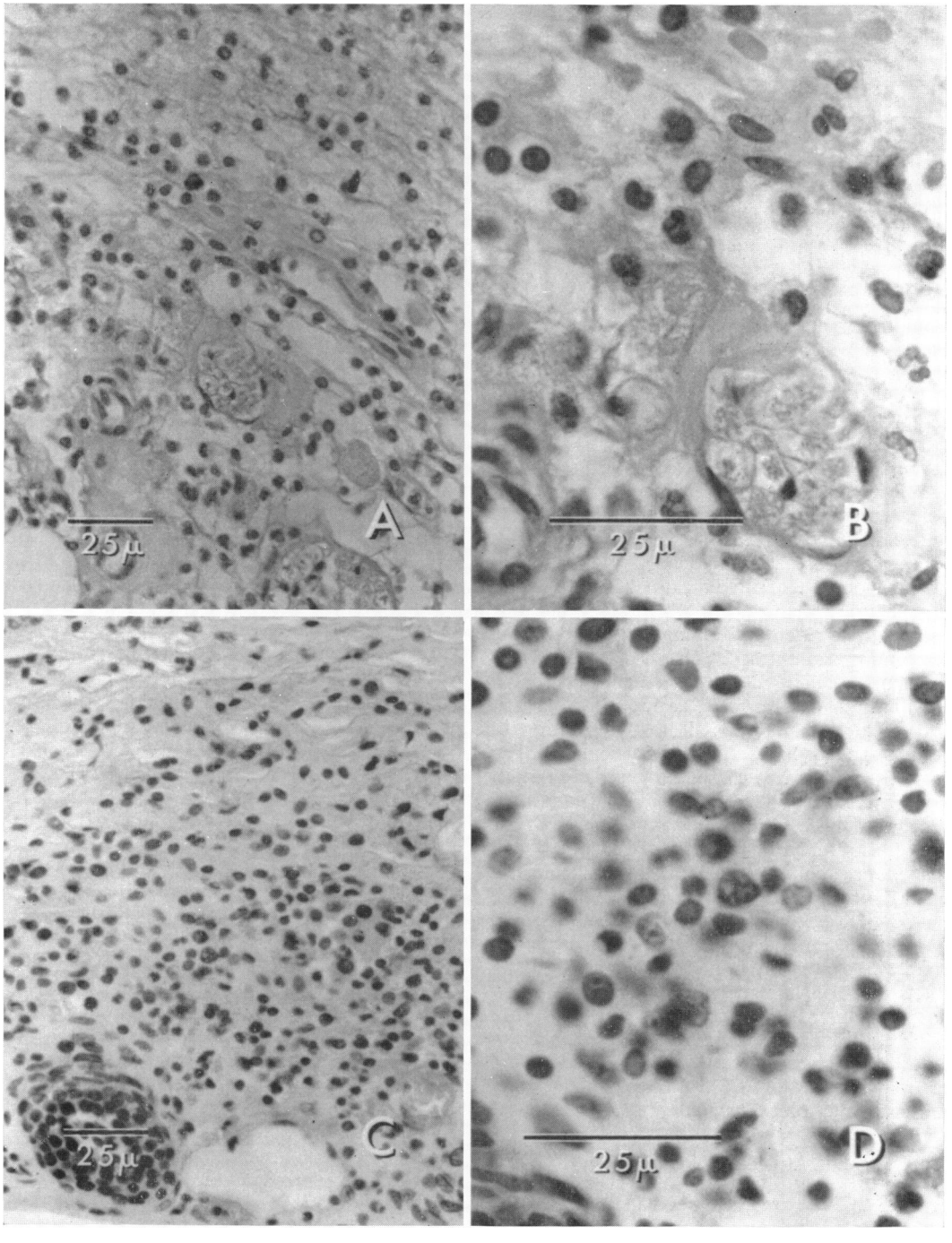

FIG. 5. *Cellular response in the footpads of spherule-vaccinated mice at 24 and 72 hr after injection of dialyzed coccidioidin. At 24 hr: (A) intensive cellular infiltration; (B) higher magnification of (A), mixture of polymorphonuclear and mononuclear cells. At 72 hr: (C) extensive cellular infiltration; (D) higher magnification of (C), mononuclear cells predominant.*

in part, to the different mouse strains or to sensitizing and testing procedures used. Also, it may be related to the concentration of antigen used to elicit the reaction; in spherule-vaccinated mice (Fig. 3), a high percentage of positive reactors was obtained only at concentration of extract >100 times that effective in man (Smith and Marcus, *personal communications*), guinea pigs (Marcus et al., Federation Proc. **24**:182, 1965) and monkeys (15). In some of our BCG-vaccinated mice, a 1:10 dilution of OT was required to elicit a positive reaction; the failure to elicit delayed reactions with PPD may have resulted from the low concentration used. In other studies, Flax and Waksman (5) reported that a concentration of tuberculin 5- to 10-fold over that used in rabbits and guinea pigs was required to elicit comparable hypersensitivity reactions in rats.

To classify a hypersensitivity reaction as the delayed type, one of the criteria often used is the demonstration of passive transfer via cells to normal animals. In spite of our inability to show such transfer (see below), several other reasons have prompted us, nevertheless, to so classify the reaction to coccidioidal extract in the footpad of spherule-vaccinated mice. Firstly, the time course of the reaction, beginning at 6 to 8 hr and reaching maximum at 24 hr after antigen injection, corresponded to that observed for tuberculin sensitivity in Namru mice, as well as other animals, and was not altered by the addition of adjuvants (Fig. 2). Although immediate swelling was observed, it was caused largely by the injection of fluid as evidenced by the reaction in nonvaccinated mice given saline. Swelling from the injection of fungal material persisted longer than that from saline injection, but usually subsided within 4 hr. Secondly, the reaction was not elicited by OT (Savage, Ph.D. thesis, Univ. California, Berkeley, 1965) and was specific for coccidioidal extracts. Finally, the cellular reaction to the extracts in vaccinated mice corresponded to that described for delayed hypersensitivity.

The histological studies showed that mononuclear cells, present at 6 hr after antigen injection, predominated at 24 to 48 hr and, by 72 hr, comprised >90% of the cellular infiltrate (Fig. 5C and 5D). Their predominance in delayed hypersensitivity reactions has been documented by many workers (7, 21). We also observed the early presence of many polymorphonuclear cells which predominated in most instances up to 24 hr (Fig. 4D, 5B). Some of them infiltrated the site in response to the fungal material, because we observed more polymorphonuclear cells in the coccidioidin-injected than the saline-injected footpad of nonvaccinated mice. The early appearance of polymorphonuclear cells in immunized mice has been noted also in delayed hypersensitivity reactions to tuberculin (1, 10, 17) and *Candida* extracts (Rowlands, Crowle, and Russe, Federation Proc. **23**:286, 1965). In addition, the extent of the polymorphonuclear infiltration has been related to the intensity of the dose-dependent reaction to tuberculin (1, 10, 21). Whether the above applies to the footpad reaction to coccidioidal extract is unknown.

We think that the early presence of numerous polymorphonuclear cells in our study is not indicative of an Arthus reaction. The animals were tested only once and at 6.5 months after vaccination, when circulating antibodies, if present, were probably at a very low level. In this regard, precipitins have been detected in spherule-vaccinated mice only after a booster dose (12), and boosters were not employed in this study. Furthermore, eosinophils, edema, hemorrhage, thrombosis, necrosis, and plasma cell types, all characteristics of the Arthus reaction (8), were either not present or present in negligible quantities. For these reasons, it is also unlikely that the mononuclear cells, predominant at 48 to 72 hr after antigen injection, represented the delayed component (8) of an Arthus reaction.

At variance with the interpretation that the hypersensitivity reaction to fungal extracts is of the delayed type, however, is the failure to transfer it passively with cells from sensitized donors (*unpublished data*). As many as 3×10^8 splenic cells from vaccinated mice were given, intravenously or intraperitoneally, or by both routes, to normal recipients either alone or with lymph node cells. Serum from vaccinated or boosted (12) mice was also transferred intravenously to other recipients. No delayed reaction was observed in recipients of either cells or serum. Vredevoe (20) also did not transfer passively a persistent induration to a protein antigen with cells, and interpreted the induration as a delayed component of the Arthus reaction which was also present. However, as mentioned before, we observed no complicating Arthus reaction in the *Coccidioides* system, and our failure to transfer passively the delayed reaction may be related to the fact that an outbred mouse strain was used. But, despite the use of inbred mice by other workers, the passive transfer of delayed hypersensitivity was successful in one study (3), but not in another (4).

Our finding that many survivors of intranasal infection with *C. immitis* arthrospores developed delayed hypersensitivity is in accord with others reported for tuberculous mice (9) and *Listeria*-infected mice (16). The possible association be-

tween delayed hypersensitivity and immunity in mice vaccinated with intact (14) and disrupted spherules (11) is under investigation.

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