Response of Hypersensitive Mice to the Footpad Injection of Living Homologous or Heterologous Mycobacteria: Preliminary Report

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Mice sensitized by the injection of viable mycobacteria into one of the hind footpads responded to a second injection of mycobacteria (3 to 4 weeks later), introduced into the contralateral foot, with a degree of footpad swelling that was both accelerated and exaggerated beyond that observed after the first inoculation. The degree of specificity of this reaction (i.e., response to homologous versus heterologous mycobacteria) was comparable to that previously reported for dermal reactions of hypersensitive guinea pigs to tuberculin or tuberculin-like antigens from mycobacteria. In preliminary studies it was impossible to achieve this state of specific sensitization by vaccinating mice subcutaneously with water-in-oil emulsions of heat-killed mycobacteria; reasons for the failure are discussed. It is suggested that this tool could prove useful in both taxonomic and immunological investigations. Advantages and disadvantages of the mouse footpad test in relation to the dermal skin test in guinea pigs are discussed.

The dermal reaction of sensitized guinea pigs to homologous and heterologous skin test antigens (4, 8, 9, 11, 13-15, 20, 23) has been proposed as one method of identifying or comparing strains or species of mycobacteria. The very impressive "sensitivity profiles" observed in hypersensitive guinea pigs by Edwards et al. (8) led to similar early studies in military personnel (2, 8) as a means of pinpointing the infecting organism in large populations. Implicit in the success of such studies, however, was the need for the "specific" tuberculin-like agent in the battery of test antigens (9); if this was missing, the infecting organism conceivably could be misidentified as the one whose skin test antigen produced the largest reaction.

During the course of a study to determine the fate of a superinfection with homologous and heterologous mycobacteria administered in the footpads of mice previously sensitized by the same route (G. P. Kubicac and F. P. Dunbar, unpublished data), the challenged footpads of homologously sensitized animals reacted both more rapidly and with significantly greater swelling than did comparable footpads challenged with heterologous mycobacteria. It seemed possible, therefore, that strains or species of mycobacteria could be compared with one another by challenging the footpads of previously sensitized mice with living bacteria. This use of viable organisms as "test antigens" also could obviate the need for specific skin test antigens, thereby eliminating the restriction that this requirement posed on the skin test in guinea pigs. This report describes our preliminary experiences with mouse footpad injection as a means of species identification.

MATERIALS AND METHODS

Mice. Specific pathogen-free female CD-1 mice (Charles River Farms), approximately 4 weeks of age, were used. They were maintained under isocaps (Carworth-Lab cages, N.Y.) and fed sterile, pelleted food and water.

Organisms. Five species of mycobacteria were investigated. Four were obtained from the Trudeau Mycobacterial Culture Collection, viz., Mycobacterium bovis BCG, strain Montreal (TMC 1012), M. kansasi (TMC 1201), M. scrofulaceum (TMC 1314), and M. fortuitum (TMC 1530). The last culture, M. intracellulare (D-673), was used according to an earlier procedure with this strain in mice (7, 19). To insure reproducibility of inocula, all cultures were grown in enriched Middlebrook 7H-9 broth (commercial source) containing 0.05% Tween 80 until the bacterial population was approximately 10⁸/ml; appropriate volumes of each suspension were bottled, frozen, and stored at -70 C to preserve viability (10).

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Representative samples of each suspension were then thawed and plate counted prior to use to determine actual numbers of viable units per milliliter.

**Experimental protocol.** Two separate experiments were conducted. In the first experiment, each of five groups of 50 mice were vaccinated in the left hind footpad with approximately $10^6$ viable units of one of the five different mycobacteria contained in 0.05 ml of 0.1% phosphate-buffered gelatin, pH 7.0. Three weeks later, the right hind footpad of each animal was measured with dial gauge calipers (Schnelltaster, Kröplin, Germany). Each group of 50 vaccinated mice was then further divided into five groups of 10 each, to be challenged in the right hind footpad by either the homologous or one of the four heterologous species of mycobacteria under test. In each instance the challenge dose was adjusted to contain $10^7$ viable units. At the same time, five similar groups of unvaccinated control mice also were challenged in the right footpad with each of the test organisms to determine the temporal contribution to footpad swelling made by the bacterial suspension itself (17).

In the second experiment, and to provide more "test sites" on each mouse, animals were immunized by subcutaneous injections of heat-killed mycobacterial cells suspended in water-in-oil emulsions. Adjuvant suspensions of seven species of mycobacteria were prepared to contain 1 mg of dead bacterial cells per ml of oil as described by Collins and Mackaness (6). The seven species studied included the five strains from the first experiment as well as *M. tuberculosis* (TMC 102) and *M. chelonae* (TMC 1544). Each of the saline-emulsified suspensions was injected twice into groups of 50 mice. The first injection was divided into two 0.1 ml doses per mouse, one into the subcutaneous tissue over each hind leg. Two weeks later, the second dose of 0.2 ml was injected into the nuchal area. Four weeks after the last injection, each group of 50 vaccinated mice was divided into five groups of 10 mice each. Three of these five groups received injections of two different suspensions of mycobacteria (approximately $10^5$ viable units), one into each of the two rear footpads; one of the injected organisms was the homologous mycobacterium. The last two groups received injections into one footpad only; one of these injections was always that of the homologous species, to determine whether or not the simultaneous injection of two species into different feet would cause a reduction in the normal response of a homologously challenged foot.

In both experiments the footpad thickness of either the vaccinated-challenged or unvaccinated-challenged control animals was measured over a period of 6 to 11 days, and results were statistically analyzed.

**RESULTS**

The many footpad measurements accrued during this investigation provided numerous combinations for statistical analyses; however, the measurements ultimately chosen for presentation were two: the first measured the increase in the size of the challenged right foot on day "n" as compared to day "0" just before challenge (i.e., Rn-R0); the second measured the difference in size of the challenged right foot of vaccinated mice (Rv) and the right foot of unvaccinated-challenged control animals (Rc), i.e., Rvc-Rc. This latter served the dual purpose of revealing (i) the early immune response, in which Rvc > Rc (and usually remained this way for homologous challenge), and (ii) revealed the later swelling of the footpad caused by the challenge organism in the right foot, which became evident from day 7 onwards (17) and especially in heterologously challenged mice, to the end that Rc > Rvc and resulted in a negative value for the plotted expression Rvc-Rc (Fig. 2).

All data were first analyzed by calculating the statistical significance of the differences between mean footpad sizes of each homologous-heterologous combination. On the assumption that appreciable error might have been introduced into our analyses by use of small groups of mice, data were also analyzed by the t-test. The reported levels of significance were apparent by both methods of analysis.

The results of experiment 1 are shown in Fig. 1 and 2. In all cases the observed measurements for average homologous reactions are shown in solid lines, whereas the range of average heterologous reactions are indicated by the broad, shaded areas. Mice vaccinated with viable *M. kansasii*, *M. bovis* BCG, and *M. intracellularare* revealed striking differences between homologous and heterologously challenged animals whether Rn-R0 (Fig. 1A, B, and C) or Rvc-Rc (Fig. 2A, B, and C) was measured. In all three cases, the differences between homologous and heterologous challenge between days 3 and 7, inclusively, are significant at probability levels ($P$) ranging from $>0.01$ to $>0.001$.

In the case of *M. fortuitum*, the observed differences between homologous and heterologous challenge organisms were not as striking. When measuring Rn-R0 (Fig. 1D), results were significant at the level of $P \approx 0.05$ on days 5 and 7, whereas at day 3 the reaction of only one mouse resulted in failure to achieve significance at the 5% level. Likewise, when measuring Rvc-Rc (Fig. 2D) results were significantly different at the 5% level on day 3 only. Hyper-reactivity in some mice challenged with *M. kansasii* caused us to just miss the 5% level of significance on days 2 and 4. Careful examination revealed that the *M. fortuitum* group of mice was vaccinated with only 5 $\times 10^5$ viable units and that, because of a dilution error, the challenge inoculum contained only $5 \times 10^4$ viable units of *M. fortuitum*, instead of the sought for 1
Undoubtedly the low numbers of viable homologous organisms used for both vaccination and challenge accounted for the poor results here (17).

Mice immunized with *M. scrofulaceum* received appropriate numbers of homologous organisms for both vaccination ($3 \times 10^6$) and challenge ($2 \times 10^6$), yet the differences recorded
between homologous and heterologous species were significant at the 5% level only on day 5 when measuring Rn-R0 (Fig. 1E). The failure to attain significance on day 3 again was due to cross-reactivity of only one species. The fact that much of the problem in this one instance was due to the actual contribution to foot thickness caused by the challenge organism itself is shown in Fig. 2E, where measurement of Rvc-Rc (where we disregarded foot increase due to challenge alone, i.e., Rc) showed a significant difference on days 1 to 3 of $P \geq 0.01$, and a 5% level of significance was observed on day 4. The contribution of cross-reactive antigens in M. scrofulaceum must also be considered and will be discussed later.

Regardless of what measurements we used, it was impossible to distinguish homologous from heterologous organisms when mice were vaccinated with heat-killed mycobacteria suspended in oil and then were challenged with viable bacilli. Here, the hoped-for advantage of two available test feet was lost when the reaction of heterologous organisms often exceeded that caused by the homologous strain. Measurements of the increase in both right (Rn-R0) and left (Ln-L0) feet for the two best and two worst oil-suspended preparations are shown in Fig. 3 and 4, respectively. The extreme cross-reactivity is evident in all four charts, and a possible reason for this will be discussed.

**DISCUSSION**

Mice, previously sensitized by injection of viable mycobacteria into one of the hind footpads, will respond 3 to 4 weeks later to a second injection of mycobacteria (homologous or heterologous) into the contralateral footpad with a degree of specificity comparable to that attained by dermal reactions of hypersensitive guinea pigs to mycobacterial antigens (4, 8, 9, 11, 13-15, 20, 22, 23). Preliminary data presented here indicate that the swelling induced by a second injection of homologous mycobacteria is statistically greater than that evoked by a heterologous challenge, suggesting that this tool could prove valuable in taxonomic as well as immunological investigations.

The advantages of using viable mycobacteria as test antigens are that: (i) there is no need for either maintenance or preparation of a battery of specific tuberculin-like antigens; (ii) there should be little concern about absorption of "test antigen" to the walls of glass or plastic containers (12). By using mice rather than guinea pigs, two additional advantages are realized: (i) more animals may be housed in much smaller space; and (ii) the draining ulcer, associated with some intradermal injections of guinea pigs (25), is avoided. On the other hand, the disadvantages must not be overlooked: (i) unpredictable numbers of mice often die from tuberculin shock within 24 h after the challenge inoculum is injected, making it necessary to sensitize a minimum of 10 mice in each study group; (ii) best results seem to be obtained when the viable count used both to sensitize and to challenge is 10^4 units/mouse; (iii) viable mycobacteria are used, thus necessitating special safety precautions for injections; and (iv) currently there is only one test site available per mouse and, hence, large numbers must be sensitized if multiple species are to be tested.

One possible way to provide more test sites per mouse would be to sensitize the animals by subcutaneous injection (in groin or nuchal area) of adjuvant-suspended, heat-killed mycobacte-
ria. In the present study it was not possible to achieve species-specific sensitization (see Fig. 3 and 4); however, this should not discourage further effort in this direction. We employed roughly 10^6 viable cells to sensitize mice via the footpad, and this represents approximately 0.001 mg of dried bacteria. In our experience (G. P. Kubica and F. P. Dunbar, unpublished data), as well as in that of others (18, 21, 24), most of the potentially pathogenic mycobacteria (except for M. leprae, M. marinum, and M. ulcerans) multiply little, if at all, after injection into mouse footpads. Apparently the bacilli persist long enough to render mice hypersensitive, but they fail to multiply significantly by the footpad route, even though the footpad may swell (17). When immunization was performed by the subcutaneous injection of heat-killed mycobacteria in oil, a total of 0.2 mg (equivalent to approximately 2 x 10^8 viable cells) of dried bacteria was injected; this was approximately 100-fold more material than was injected with living cells. In view of reports of common protein antigens fractionated from mycobacteria (1, 3, 16), it is conceivable that the extensive cross-reactions in mice sensitized with dead bacilli could have been due to introduction of 100-fold-larger amounts of common cross-reactive antigens.

Attempts to immunize mice by the intravenous injection of viable mycobacteria were discouraged because Collins had reported (5) that certain strains of mycobacteria, other than tubercle bacilli, which failed to multiply after intravenous injection also failed to induce either sensitivity or immunity. This observation also was reported for certain drug-resistant strains of bacille Calmette-Guérin (5), whereas the same organism given subcutaneously in oil gave both delayed hypersensitivity and protection (F. M. Collins, Amer. Rev. Resp. Dis., in press).

The extensive cross-reactivity seen in M. scrofulaceum-sensitized mice (Fig. 1E), which made specific identification difficult, would corroborate earlier reports of such cross-reactivity by Edwards et al. (8, 9). Whether this cross-reactivity is strain related must be ascertained by further testing, and certainly this must be done because Chaparas et al. (4) reported the skin test agent from M. scrofulaceum to be one of the least cross-reactive antigen preparations they used.

Although most of the footpad measurements revealed statistically significant differences between species for a period of 3 to 5 days after injection of challenge organisms, it would be technically more acceptable if only one time period were used for measurement; this could be possible after analysis of more extensive data to determine the specificity of footpad reactions when multiple strains of both homologous and heterologous mycobacteria are studied. In addition, further attempts must be made to provide more “test sites” per animal, and this could involve: (i) reinvestigation of oil-suspended, heat-killed vaccines; (ii) a search for strains of each species of Mycobacterium which is capable of immuno-stimulation after intravenous injection (5); or (iii) examination of still other routes of immunization.

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