Laboratory Procedures for Potency Testing of Blastomycin, Histoplasmin, and Coccidioidin (36502)

S. D. CHAPARAS, V. J. FULLER, AND E. B. SELIGMANN
(Introduced by H. Baer)

Division of Biologics Standards, National Institutes of Health, Public Health Service, U.S. Department of Health, Education and Welfare, Bethesda, Maryland 20014

Standarization of skin test reagents is of utmost importance in studies designed to assess the significance of positive reactions. Ideally, a standard should be prepared in a relatively large batch and dispensed freeze-dried in precisely measured aliquots of concentrate. The quality of such a batch may be assessed very carefully in large numbers of human beings in geographic areas where the product is to be used. Logically, any new lot of diagnostic substance for dermal tests destined for use in human beings should first be evaluated in suitably sensitized laboratory animals. Tests in animals may be used to verify that each lot prepared from the concentrate is of the desired potency. Because the guinea pig develops a high level of delayed sensitivity to many substances and has been shown to be useful for the assessment of skin reactive potency of fungal reagents (1–4), we used this species to investigate the effects of various procedures in the induction of sensitivity to histoplasmin, blastomycin, and coccidioidin.

Materials and Methods. Skin test reagents were reference materials used currently in the United States for potency assays of licensed fungal reagents. Histoplasmin was Lot No. H-42, blastomycin was Lot B8, and coccidioidin was Lot 64D2.5. *Histoplasma capsulatum* and *Blastomyces dermatitidis* used in sensitizing preparations were obtained from the surface of Long's synthetic medium (5) as mats which consisted of mycelia and spores and are referred to as spore-mat preparations. *Coccidioides immitis* spores were obtained in spherule form after growth in Converse medium with 0.05% Tween 80 (6). All spore and spore-mat preparations were autoclaved at 121° for 15 min and are referred to as "S." For sensitization of guinea pigs, the following materials were administered subcutaneously in 0.2 ml as water-in-oil emulsions in the nuchal area: (1) S alone, (2) S plus heat-killed mycobacteria (*Mycobacterium bovis*, BCG), (3) skin test material alone, (4) skin test material plus mycobacteria, (5) S and skin test material, (6) S, skin test material and mycobacteria, and (7) mycobacteria alone. Spore preparations were used at a final concentration of 20 mg/1.0 ml. The water-in-oil emulsions consisted of equal parts of saline phase which contained the skin test material and of oil phase (Arlacel A:Drakeol 6VR;35:65) which contained the S and the mycobacteria. Mycobacteria were used at 25 mg/1.0 ml. The potency of the reference skin test materials used for sensitization varied, but these were used in their available undiluted form. Skin testing was performed 6 weeks after sensitization and the following dilutions were determined to be suitable for use: Histoplasmin 1:25, 1:50, and 1:100; Blastomycin 1:200, 1:400, and 1:800; Coccidioidin undiluted, 1:2, and 1:4. These dilutions were administered intradermally in a 0.1 ml volume in all guinea pigs as homologous and heterologous challenges. Reactions were read as two perpendicular diameters of erythema at 24 hr. The results are based on two experiments consisting of a total of 24 guinea pigs for each reaction.

Results. Results of skin tests and slopes of curves were analyzed by the F test and are shown in Figs. 1, 2, and 3. The combination

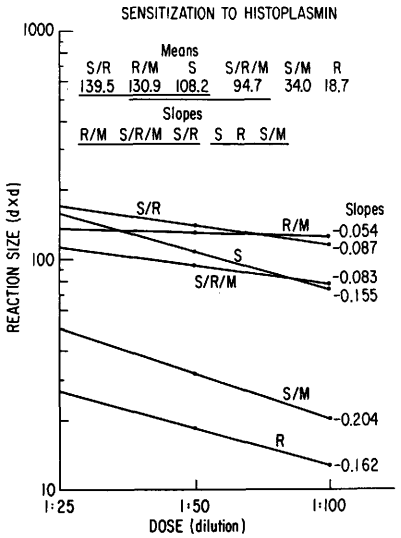

FIG. 1. Geometric mean dose response curves in guinea pigs sensitized to histoplasmin. S = spore or spore-mat preparation; R = skin test reagent; M = mycobacterial adjuvant. Comparison of means of the three dilutions and slopes were done according to Tukey [cited in Ref. (7)]. Sensitizing regimens not underscored by a common line are different at the 5% level.

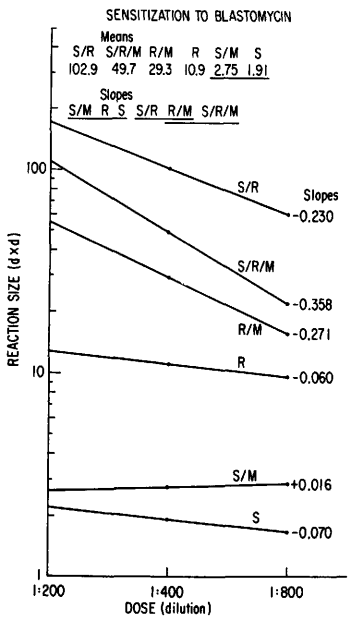

FIG. 2. Geometric mean dose response curves in guinea pigs sensitized to blastomycin. For explanation of terms see legend of Fig. 1.

of ingredients for the most effective sensitizing preparation for each of the skin reactive materials varied. For example, for histoplasmin sensitization three combinations were equally effective in inducing a high degree of sensitivity: emulsions with (a) spore-mat and histoplasmin, (b) spore-mats alone, or (c) histoplasmin with mycobacteria (Fig. 1). For blastomycin sensitivity a combination of spore-mat and blastomycin were the most effective (Fig. 2). For coccidioidin, emulsions with spherules, coccidioidin, and mycobacteria combined were the most effective (Fig. 3).

For histoplasmin sensitizations (Fig. 1), emulsions of histoplasmin alone in these experiments induced very little sensitization. The addition of mycobacteria to histoplasmin emulsions induced excellent sensitization. On the other hand, emulsions of spore-mat histoplasma alone induced very good sensitization. However, the incorporation of mycobacteria to spore-mat emulsions markedly reduced the degree of sensitization. Incorporation of mycobacteria to emulsions of histoplasmin and spore-mat combined also lowered the sensitivity.

Sensitivity to blastomycin (Fig. 2) with emulsions of either spore-mat alone or blastomycin alone induced either no or very poor sensitivity to blastomycin. When these two reagents were combined, a high degree of sensitivity was induced. Addition of mycobacteria to these effective emulsions caused a decrease in the degree of sensitivity. Addition of mycobacteria to the emulsions with blastomycin alone caused a fair degree of sensitization, but mycobacteria added to emulsions of spore-mat alone had no effect on blastomycin sensitization.

Addition of mycobacteria to emulsions containing coccidioidin enhanced the level of sensitivity to this substance. However, mycobacteria added to emulsions of spherules alone did not increase the level of sensitivity over that of spherules alone.

Cross reactions between histoplasmin, blastomycin, and coccidioidin were always less than 36 mm^2. Thus, a great deal of specificity was induced by the sensitization pro-

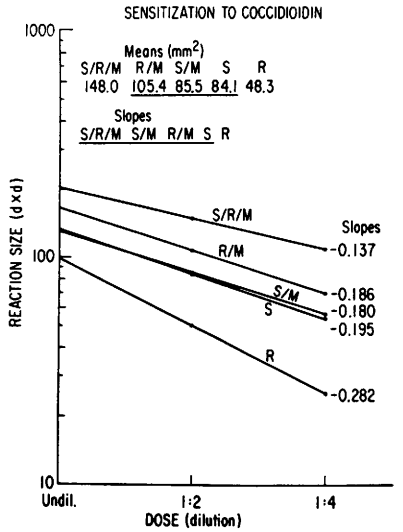

FIG. 3. Geometric mean dose response curves in guinea pigs sensitized to coccidioidin. For explanation of terms see legend of Fig. 1.

cedure.

Discussion. As has been shown (1–4) and reproduced here, it is possible to induce high levels of sensitivity to diagnostic substances for dermal tests in guinea pigs with the use of heat-killed organisms or their products. However, the level of sensitization may vary widely depending on the combination of ingredients in a sensitizing preparation. Besides the degree of sensitivity induced, the slope of dose–response curve is an important consideration in potency assays. A steep slope may be more desirable than a flat slope. For example, in the induction of sensitization to histoplasmin (Fig. 1) three regimens were equally effective: spore-mat plus histoplasmin (S/R), histoplasmin plus mycobacteria (R/M) and spore-mat alone (S). However, the slope of the dose–response curve for S was steeper which makes this procedure of sensitization more suitable for potency assays.

Not all dermal reagents are equally effective antigens, either from the standpoint of the induction of hypersensitivity or the elicitation of a skin reaction. Although the U.S. reference reagents are each used at a dilution of 1:100 for skin testing human beings, the ability of these reagents to induce sensitivity or react in guinea pigs varied considerably. For example, in the most reactive groups of blastomycin-sensitive guinea pigs, blastomycin at dilutions of 1:200, 1:400, and 1:800 elicited skin reactions of the same order of magnitude as coccidioidin used undiluted and at dilutions of 1:2 and 1:4 in guinea pigs optimally sensitized to coccidioidin. Yet emulsions of undiluted blastomycin alone did not induce a significant level, while those of undiluted coccidioidin alone did. Obviously, extensive studies on effects of dose are warranted.

Emulsions of blastomycin alone or blastomyces spore-mats alone were ineffective, and yet when these two substances were combined, they were most effective. There are several possible reasons for this effect. The washed blastomyces spore-mat might be low in skin reactive antigens found in blastomycin, which might account for the inability of the spore-mat preparation alone to sensitize. Blastomycin itself must be assumed to be a poor, possibly tolerogenic, antigen since it alone could not induce significant sensitivity. However, good sensitivity occurred when blastomycin was combined with the spore-mat presumably via "schlepper" action or adjuvant action induced by blastomyces spore-mat in a manner similar (in this case, better) to mycobacteria.

A double-edged effect of mycobacteria was observed. In some situations, addition of mycobacteria to sensitizing emulsions caused an increase in the level of sensitization, but in others a decrease was observed. In these studies, when a preparation caused a level of sensitivity such that the largest skin test dose induced a reaction less than 100 mm^2, the addition of mycobacteria to the sensitizing preparation caused an increase in the level of sensitivity. The ineffectiveness of mycobacteria in spore-mat preparations of blastomyces is possibly due to the absence of appropriate antigen from this preparation as discussed above. On the other hand, if a preparation induced a level of sensitivity such that the largest skin test dose gave a reaction size greater than 150 mm^2, the addition of mycobacteria to the sensitizing preparation

caused a decrease in the level of sensitivity. No significant increase or decrease was seen by the addition of mycobacteria to preparations that induced reactivity such that the largest skin test dose induced reaction sizes between 100–150 mm^2. The decrease in reactivity may be best explained on the basis of antigenic competition. Competition was seen only when the sensitizing preparation was a good inducer of sensitivity. If the sensitivity preparation was minimally effective, then adjuvanticity was seen rather than competition.

More detailed work needs to be accomplished in order to elaborate the effects of dose of each substance in a sensitizing mixture and to explain fully the tolerogenic, competitive, or adjuvant effects of ingredients in sensitizing preparations. Nevertheless, the results presented emphasize that variations in the composition of ingredients in preparation for sensitization to fungal antigens can cause profound difference in levels of reactivity of animals to these reagents.

Summary. Ways to induce sensitization in guinea pigs for use in assays for histoplasmin, blastomycin and coccidioidin were investigated, using various combinations of spore preparations, skin reactive reagent and mycobacteria incorporated in water-in-oil emulsions. It was possible to induce high levels of sensitivity to the skin reactive reagents, but the level of sensitization varied widely, depending on the combination of ingredients in a sensitizing preparation. The addition of mycobacteria sometimes caused either a decrease or an increase in the level of sensitization.

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