

Preferential development by mice of delayed hypersensitivity to purified basic proteins

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Mice injected with purified protein antigens (e.g., serum albumins) in incomplete Freund adjuvant develop both Arthus and delayed hypersensitivities. The presence of the former interferes with observation of the latter. But mice similarly injected with methylated derivatives of such purified proteins preferentially develop delayed hypersensitivity. Hence the latter can be studied independently of a preceding Arthus reaction. Such use of methylated serum albumins in the experiments reported here showed that polymorphonucleocytes outnumber monocytes early in specific delayed hypersensitivity skin reactions but later are replaced by large numbers of monocytes, that passive transfer of delayed hypersensitivity may require cooperation between lymphoid cells and humoral antibody, and that immediate and delayed hypersensitivities in mice can be distinguished from each other by differences in antigenic determinant sites. Methylated serum albumins are easy-to-prepare basic proteins which are readily soluble at acid but not alkaline pH.

Although the advantages of using widely available purified protein antigens (e.g., ovalbumin, serum albumins) in research on delayed hypersensitivity are many,^{1, 2} these induce Arthus hypersensitivity so readily that distinguishing between the effects of humoral antibody hypersensitivity and delayed hypersensitivity has been difficult¹⁻⁷; special techniques have had to be devised to induce the latter without the former. For instance, the injection of minute quantities of antigen, of appropriate antigen-antibody complexes, or of partially denatured antigen are methods which have been used frequently in the guinea pig.^{2, 8-10} So far, they have not worked in mice.^{5, 11, 12} When these animals are injected with one of the commonly used purified proteins in incomplete Freund

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adjuvant for the induction of delayed hypersensitivity, they invariably develop strong enough Arthus hypersensitivity¹³⁻¹⁵ to suggest that what appear to be delayed hypersensitivity reactions^{10, 11, 16, 17} may actually be prolonged Arthus reactions.^{3, 5} Although we have induced delayed hypersensitivity to polysaccharides in mice without accompanying Arthus hypersensitivity,¹⁸ and other workers have found evidence that mice can develop the one without the other to various other microbial antigens,¹⁹⁻²² there has remained an urgent need for a technique of inducing uncomplicated delayed hypersensitivity in mice to common, purified protein antigens.

We have discovered that mice preferentially develop delayed hypersensitivity to methylated foreign serum albumins. In addition to supplying an answer to the need just mentioned, this discovery has begun to lead to valuable accessory data, such as indicating that in mice, as is usually also true of guinea pigs,^{2, 8} antigenic determinant groups for humoral and cellular antibodies differ. The following report²³ describes preparation of methylated serum albumins, their use for inducing delayed hypersensitivity in mice, and some of the characteristics of interaction between these antigens and mice sensitized to them or their native precursors.

MATERIALS AND METHODS

Antigens

Crystalline-grade bovine and human serum albumins (BSA and HSA, respectively) were purchased from Pentex, Incorporated (Kankakee, Illinois). Methylated serum albumins (MeSA) were prepared from these as described below.

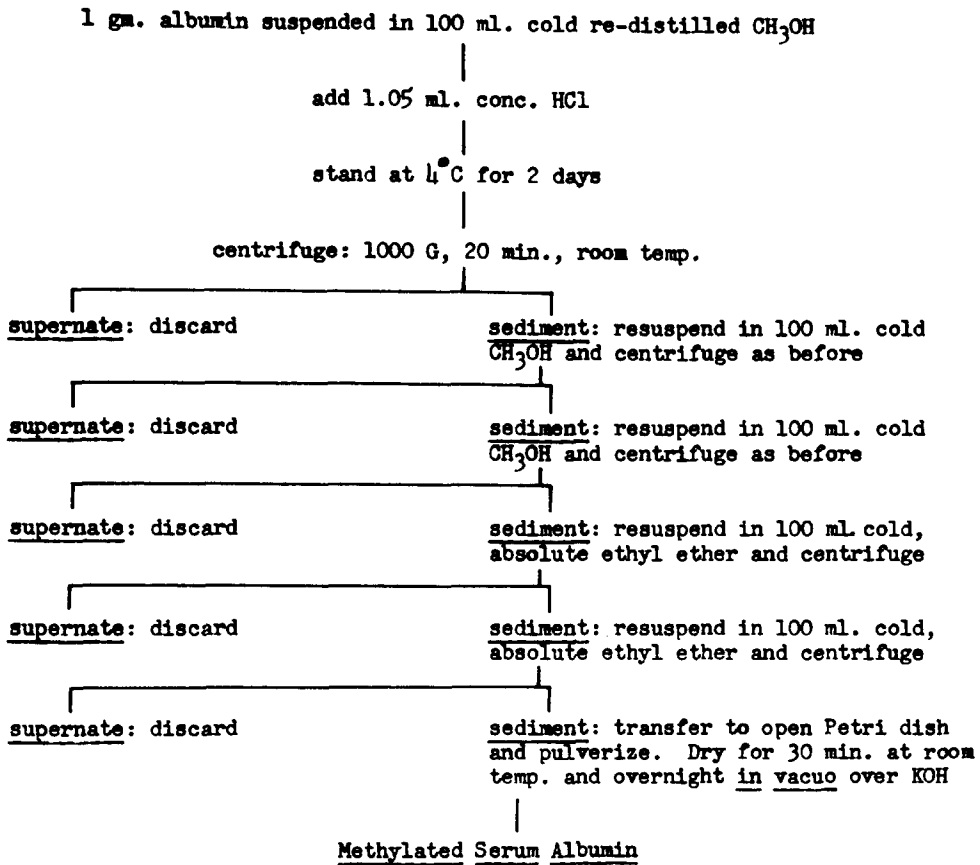
Preparation of methylated albumins

The following method was developed from that mentioned by Mandell and Hershey.²⁴

One gram of albumin is suspended in 100 ml. of re-distilled methanol previously chilled to approximately -29° C., and 1.05 ml. of concentrated hydrochloric acid is added (Fig. 1). This results immediately in a clear solution. The mixture, which becomes turbid once again with precipitating gelatinous MeSA within a few hours, is kept at 4° C. in a refrigerator for 2 days to ensure complete methylation. The precipitate is centrifuged out, resuspended twice in prechilled methanol and then twice more in prechilled absolute ether. Then it is transferred to an open Petri dish, where it is pulverized with a glass rod, dried at room temperature for 30 minutes and then overnight *in vacuo* over KOH. Since the product, a heavy, finely granular white powder, is readily soluble in distilled water and only slowly so in buffered physiologic saline (it is insoluble in saline buffered to pH 7.4), for *in vivo* use it is dissolved in distilled water and made physiologic by adding solid NaCl.

Experimental animals

CF-1 female mice (Carworth Farms) were used in groups of 10 and were maintained on water and Rockland mouse food.

**Fig. 1**

Flow sheet outlining preparation of a methylated serum albumin.

Sensitization and testing

For sensitization, each mouse was injected subcutaneously on days 0 and 7 with 0.1 ml. volumes of water-in-oil emulsion (incomplete Freund adjuvant) containing 0.25 mg. of antigen. Hypersensitivity was detected by intradermal tests with 0.02 ml. volumes of 0.1 per cent antigen in unbuffered saline; these tests were given on alternating flanks on days 21, 35, and 49. Generally, reactions were read at 3 and 24 hours after skin-testing, although more frequent readings sometimes were taken as indicated further on. More complete details and information on our techniques for sensitizing and testing mice can be found in references 25 and 26.

Microscopic sections

The histopathology of mouse dermal reactions to methylated albumins was followed by examining paired skin biopsies taken at various intervals from different but similarly sensitized and tested mice, fixed in 10 per cent buffered

formalin, sectioned, and stained with hematoxylin-eosin (courtesy of Dr. Phyllis Kind, Department of Pathology, University of Colorado Medical School).

Passive transfer

Donors, proved hypersensitive by skin tests performed several weeks before their use, were bled out from severed neck vessels into a beaker; their spleens were removed and pooled in Hanks solution kept at room temperature. Ten donors were used for 10 recipients. The pooled bloods were rimmed, after coagulation, and incubated at 37° C. for 1 hour; serum was harvested by centrifugation. The pooled spleens were rinsed with Hanks solution and then pressed with a glass pestle through a fine-mesh Monel metal screen. Cells in the resulting homogenate were suspended by sucking through a 22 gauge needle into about 10 ml. of Hanks solution, centrifuged out at 1,000 G for 15 minutes, and resuspended with 2 ml. of Hanks solution. Packed cells from 10 donor mice averaged 0.7 ml., of which 0.2 ml. was buffy coat. Each recipient was injected with 0.2 ml. of the resulting 2.7 ml. suspension and therefore received approximately 0.05 ml. of packed cells; 80 to 90 per cent of nucleated cells in these suspensions were viable by the eosin exclusion test.

Cells and serum were injected intravenously within less than 2 hours after donors had been sacrificed; when both were used, serum (0.2 ml. per mouse) succeeded cells. Recipients were skin-tested a few minutes either before or after transfer.

Passive cutaneous anaphylaxis

Donor serum was diluted severalfold with pH 7.4 physiologic phosphate buffer, and each dilution was injected intracutaneously in 0.02 ml. volumes into each of 2 normal recipient mice. Three to 4 hours later each such mouse received intravenously 0.2 ml. of a combination of 0.5 per cent antigen and 0.5 per cent Evans blue dye. Reactions were read 20 to 40 minutes later by right-angle measurements of bluing diameter.

Systemic hypersensitivity

Systemic hypersensitivity reactions were observed in connection with experiments on the production of serum contrasensitizer.²⁷ The production of this substance, not to be detailed in this paper, is induced in hypersensitized mice by injecting them intraperitoneally with 0.1 mg. of antigen in saline and following this 1 and 2 days later with injections, respectively, of 1.0 and 10.0 mg. of the same antigen. Highly sensitized mice will develop good systemic reactions to the lowest dose; moderately sensitized animals may not react obviously until receiving the second dose. Both anaphylactic and delayed hypersensitivity reactions can be observed. They were graded subjectively by the general appearance of the animal at 45 minutes (anaphylactic) or 24 hours (delayed) and objectively by the degree of hypothermia that they experienced at these intervals,^{28, 29} as measured rectally with a Tri-R Electronic Thermometer.

Gel electrophoresis and immunodiffusion

Electrophoretic characteristics of the methylated albumins were compared with those of native albumins under various conditions.³⁰ For the example presented here, 1 per cent solutions of MeHSA and HSA were electrophoresed simultaneously on a single microscope slide through 2 per cent agarose (Sea-Kem) in pH 7.0, 0.01M, Sorensen phosphate buffer for 15 minutes at 75 volts. Migration of MeHSA was visible directly because it precipitated spontaneously; the spot of electrophoresed HSA was detected by soaking the slide in 95 per cent ethanol for a few minutes. Immunodiffusion analyses were performed in 2 per cent gelatin (Difco-Bacto) made up in 0.1M Sorensen phosphate buffer of pH 6.5.³⁰

EXPERIMENTS AND RESULTS

Preparation and characteristics of methylated serum albumins

The procedure described above for preparing MeSA (Fig. 1) has been reliable and provides altered antigens of predictable characteristics. These characteristics readily distinguish the basic MeSA from its acidic predecessor. Thus, contrary to the latter, MeSA is more soluble at acid than at alkaline pH, and it is attracted electrophoretically toward the cathode rather than the anode (Fig. 2). MeSA dissolved in distilled water is precipitated rapidly from solution by ammonium hydroxide, but it stays in solution when either acetic or trichloroacetic acid is added.

Sensitizing properties

MeBSA and MeHSA readily induce delayed-type hypersensitivity in mice while at the same time inducing little or no Arthus hypersensitivity. This contrast with the behavior of the corresponding native serum albumins is illustrated in Table I, which shows that both native and methylated albumins induced delayed hypersensitivity well. But whereas all mice receiving either kind of native albumin had developed Arthus hypersensitivity within 3 weeks and retained it throughout the experiment, neither group of mice receiving methylated analogues had developed any 3 hour reactivity at 3 weeks. Some

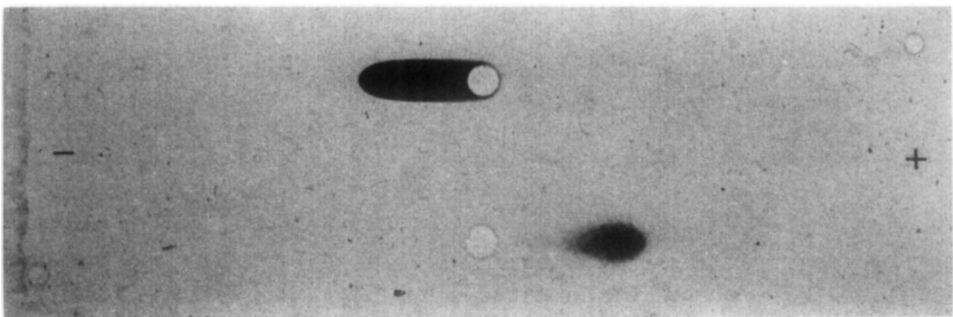


Fig. 2

Agarose electropherogram comparing migrations of MeHSA (above) and HSA (below) at pH 7.0.

of the latter mice, especially those receiving MeBSA, did develop an earlier-appearing reaction late (at 8 weeks) in the experiment. Although these may have been Arthus reactions, they were much weaker than the 24 hour reactions which succeeded them and never showed the petechiae commonly seen for 3 hour reactions to the native albumins.

Macroscopic appearance of the typical skin reaction

One hour after mice sensitized with a methylated albumin have been skin-tested with this antigen, the test site is a small edematous bleb; within another hour this usually disappears. Occasionally, this bleb remains and by the third hour becomes slightly indurated. Neither erythema nor petechiae appear in such instances, but the center of the reaction becomes somewhat blanched. At 5 or 6 hours mice hitherto without reaction begin to develop a small, hard nodule with a pale center; and in those with the earlier reaction, the inflammation becomes indurated and blanching more obvious. At 24 hours, as determined by examining these reactions macroscopically, using sacrificed, skinned mice, the reaction consists of a mound of infiltrating cells about 6 mm. in diameter surrounded by a narrow zone of hyperemia, which itself is surrounded by a broad, rather indefinite zone of edema; total diameter of the reaction averages about 1 cm. (Figs. 3 and 4). Intense 24 hour reactions sometimes are erythematous.

Over the next day, edema diminishes, the mound of infiltrating cells flattens and broadens, and the reaction center becomes necrotic and pitted. At 7 days the reaction, though much decreased, remains readily palpable in most mice and has an eschar at its center.

In unsensitized mice, the bleb caused by testing disappears within 1 to 2 hours, and no additional reaction develops.

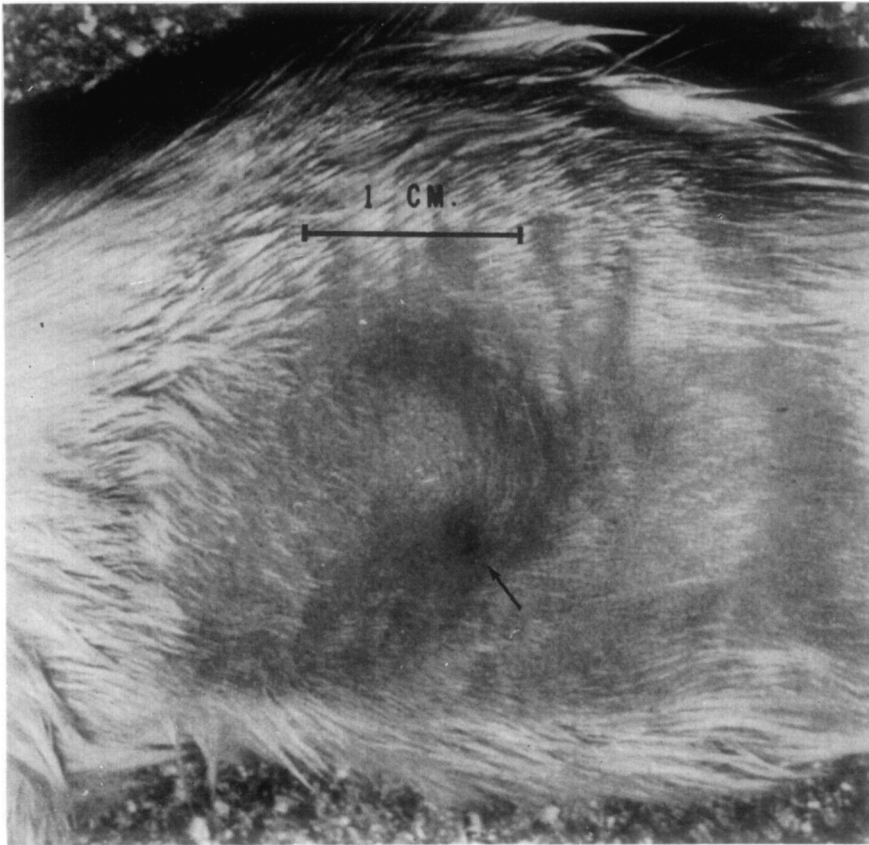
Microscopic development of the reaction

Examination of a series of succeeding biopsies made in pairs from skin reactions in a number of equally sensitized and challenged mice revealed the

Table 1. Hypersensitivities induced in mice by native and methylated serum albumins

Vaccine	Test antigen	Test time*					
		3		6		8	
		I	D	I	D	I	D
Emulsion	BSA	13	0	0	0	50	0
Emulsion	HSA	0	0	10	0	20	0
Emulsion	MeBSA	0	0	0	82	0	82
Emulsion	MeHSA	0	0	0	20	0	0
BSA	BSA	100	90	100	80	100	90
MeBSA	MeBSA	0	100	10	90	60	60
HSA	HSA	100	90	100	100	100	80
MeHSA	MeHSA	0	70	0	100	10	100

I and D represent immediate and delayed reactions; results are given as per cent of mice reacting in groups initially of 10 mice each.
*Test time indicated in weeks after first vaccination.

**Fig. 3**

Appearance of typical dermal reaction to MeHSA in MeHSA-sensitized mouse 24 hours after skin-testing. Animal was skin-tested 7 weeks after original immunization; scar remaining from 3 week test done in same area also is visible (arrow).

following microscopic events. Within 3 hours, polymorphonucleocytes begin to accumulate, some within the fatty area but most bordering above and below the subcutaneous layer of muscle. These cells tend to be clustered around blood vessels. At this time monocytes and lymphocytes are few, numbering only 1 monocytic cell for 20 polymorphonucleocytes. But over the next 3 hours this proportion changes; at 6 hours it has become 1:3. The total number of infiltrating cells at this time is equal to or less than that at 3 hours, and their distribution remains the same. By 9 hours after challenge the proportion of polymorphonucleocytes to monocytes has become nearly equal, the total number of cells has diminished, and these have become more evenly distributed, with less perivascular congregation.

During the next several hours, a second and considerably heavier wave of cellular infiltration arises, so that at 24 hours there is a very dense massing of these cells, this once again centering around blood vessels. However, by

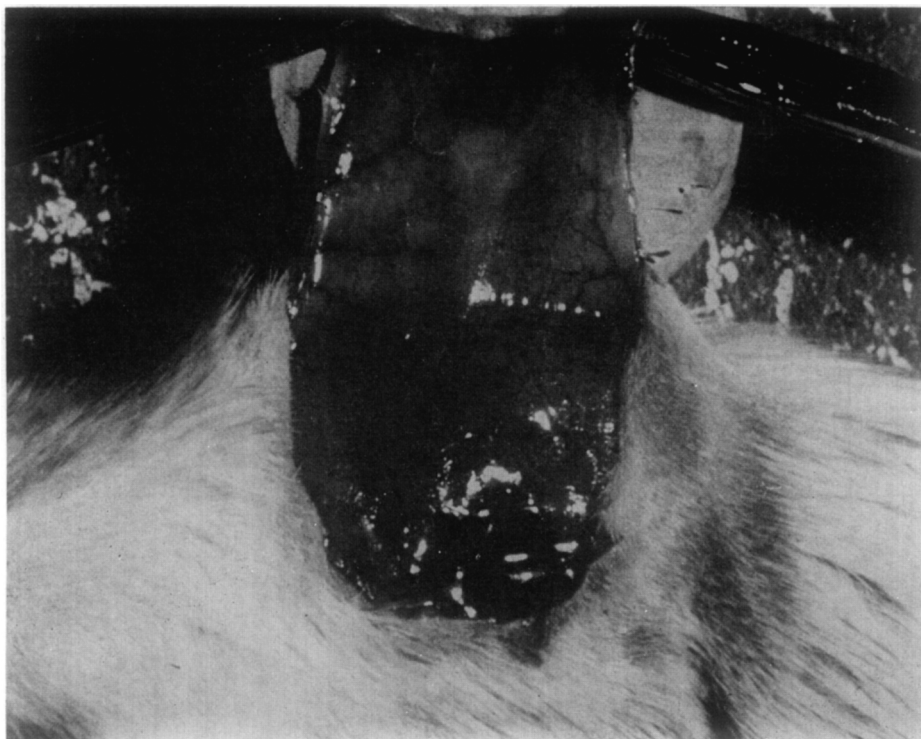


Fig. 4

Subcutaneous view of 24 hour delayed hypersensitivity dermal reaction to MeHSA in MeHSA-hypersensitive mouse.

now many are found below the muscle layer. Monocytes outnumber polymorphonucleocytes 2:1 over-all, and this proportion is 4:1 beneath the muscle. Subdermis and dermis also have become lightly infiltrated. At 48 hours, some infiltrating cells remain within or near to the dermis, but most now are beneath the layer of muscle, still prominently perivascular. Over-all, monocytes outnumber polymorphonucleocytes 4:1, two thirds of the monocytes appearing to be small lymphocytes.

From 48 hours through day 7 the number of infiltrating cells gradually decreases, apparently by degeneration and lack of replacement by newly infiltrating cells.³³ The cells become scattered and no longer concentrated around blood vessels. Most remaining cells are either small lymphocytes or fibroblasts; the previous cellular infiltration now is being replaced with amorphous eosinophilic substance and fibrin.

These reactions continually evince mild edema. As suggested previously in this paper, they develop in two waves, one starting before 3 hours and declining shortly thereafter and the other beginning after 12 hours and reaching a peak at 24 hours.* The second is a considerably more severe reaction

*The time at which the second wave begins depends upon the degree of hypersensitization; in highly sensitive mice this may be as early as 5 or 6 hours.

Table II. *Passive cutaneous anaphylaxis tests*

<i>Antiserum</i>	<i>Test antigen</i>	
	<i>HSA</i>	<i>MeHSA</i>
To MeHSA	64*	64
To HSA	512	8
Normal serum	0	0

*Reciprocal of greatest dilution showing bluing. Dilution end points were sharp, differences between reaction and no reaction being 8 to 9 mm. of bluing vs. none at all. Sera used were pools from the bleedings of several mice.

Table III. *Anaphylactic and delayed-type systemic reactions in mice sensitized and challenged with native and methylated albumins*

<i>Sensitizing antigen</i>	<i>Challenge antigen</i>			
	<i>HSA</i>		<i>MeHSA</i>	
	<i>0.1 mg.</i>	<i>1.0 mg.</i>	<i>0.1 mg.</i>	<i>1.0 mg.</i>
<i>Anaphylactic</i>				
HSA	3+	4+	±	+
MeHSA	±	2+	±	2+
<i>Delayed</i>				
HSA	-	-	±	+
MeHSA	-	-	±	2+

Ratings are presented on a 0 to 4+ scale based on grossly visible reactions, relative hypothermia, and on proportion of challenged mice dying; 1.0 mg. doses of MeHSA caused positive and negative reactions in unsensitized mice.

than the first and declines very slowly over several days. The first wave of reaction usually is not detectable grossly, and it does not interfere with study of the second wave as does the severe Arthus reaction which develops in mice sensitized and tested with native albumins. Whether or not this first wave signals a mild Arthus reaction, its occasional presence suggests that mice can make a moderate humoral antibody response to MeSA. We sought direct, non-microscopic evidence for this by using the passive cutaneous anaphylaxis test.⁹

Passive cutaneous anaphylaxis

Pairs of mice injected intradermally with doubling dilutions of antisera obtained from either HSA- or MeHSA-sensitized mice and challenged intravenously 2.5 hours later, as described in Materials and Methods, with a mixture of antigen and Evans blue dye,* provided the results summarized in Table II. These show that mice sensitized with MeHSA developed moderate titers of humoral antibodies which reacted indiscriminately with both MeHSA and HSA. Mice immunized with HSA developed high titers of passive cutaneous anaphylaxis (PCA) antibodies against this albumin but only low reactivity to MeHSA.

*When Evans blue was added to the solution of MeHSA in physiologic unbuffered saline it formed a fine precipitate, apparently because of development of an insoluble dye-antigen complex.

Systemic reactions

Understanding of systemic anaphylaxis reactions in mice is poor and of systemic delayed hypersensitivity reactions inferior even to that. In connection with other experiments, we have observed some of the conditions needed to elicit these with native and methylated serum albumins. The results of some of these observations are included here because, though they are perfunctory, they contribute to a more complete description of the relative reactivities of mice to native and methylated albumins. Summarized in Table III by an arbitrary scale of 0 to 4+, they indicate that MeHSA induces a weak anaphylactic hypersensitivity, detectable with both HSA and MeHSA, and that HSA induces a strong anaphylactic sensitivity to itself but only a weak one to MeHSA. These results fully confirm those of the PCA tests (Table II). In addition, the data in Table III indicate that MeHSA induced modest systemic delayed hypersensitivity specific for itself. No such reactions were seen in mice sensitized and tested with HSA, although mice sensitive to HSA did seem to react slightly with MeHSA.

Passive transfer of hypersensitivity

By its various characteristics the principal skin reaction in mice sensitized and challenged with methylated serum albumins appeared to be due to delayed hypersensitivity and thus should be transferable passively with lymphoid cells but not serum. As will be seen, this expectation was only partially borne out.

Bloods and spleens from each of 20 hypersensitive donor mice were collected and pooled. Serum and cells were prepared from these as described in Materials and Methods. Of 30 normal recipients which were being skin-tested during the transfer procedures, 10 received cells, 10 received serum, and 10 received both cells and serum. The results from this experiment are summarized in Table IV.

These show that 3 hour reactivity was transferred into a few recipients with antiserum, but not cells, that no 24 hour reactivity was transferred by antiserum alone, that cells alone transferred neither reactivity, and that cells injected along with antiserum transferred both types of reactivity. Thus, lymphoid cells are needed for passive transfer of delayed hypersensitivity to MeHSA, but under the conditions of these experiments (e.g., skin-testing at the time of transfer) antiserum also is required.

Skin-test cross-reactivities of native and methylated albumins

Since mice immunized with MeHSA seemed to be making some humoral antibodies to it, their negligible development of Arthus reactivity might be an artifact; for instance, by combining with skin proteins MeHSA could have its antigenic determinants for Arthus antibodies blocked. To investigate such possibilities, a cross-test of mice sensitized with native or methylated albumins was made with groups of 10 mice sensitized 5 weeks earlier and tested only once. The results, summarized in Table V, disclose that both MeBSA and MeHSA induce delayed hypersensitivities specific for themselves: neither native albumin elicited any delayed hypersensitivity reactions in mice sensi-

Table IV. *Passive transfer of delayed hypersensitivity in mice to MeHSA*

<i>Material transferred</i>	<i>Time (hours)</i>	<i>Individual reaction diameter* (mm.)</i>	<i>% Reacting</i>
Serum	3	1, 1, 6, 1, 7, 1, 1, 7, 1, 1	30
	24	1, 1, 1, 1, 1, 1, 1, 1, 1, 1	0
Cells	3	1, 1, 1, 1, 1, 1, 1, 1, 1, 1	0
	24	1, 1, 1, 6, 1, 1, 1, 1, 1, 1	10
Serum and cells	3	7, 6, 1, 8, 9, 9, 8, 1, 6	78
	24	6, 5, 6, 5, 5, 4, 5, 6, 5	100

*Diameter of swelling. When no reaction was evident a mouse was assigned a reading of 1 for statistical purposes.

Table V. *Skin test cross-reactivities in hypersensitive mice to native and methylated serum albumins*

<i>Sensitizing antigen</i>	<i>Test antigen</i>	<i>% Reacting</i>	
		<i>I</i>	<i>D</i>
Emulsion	BSA	0	0
Emulsion	MeBSA	0	0
Emulsion	HSA	0	0
Emulsion	MeHSA	0	0
BSA	BSA	100	100
BSA	MeBSA	10*	70
MeBSA	BSA	10*	0
MeBSA	MeBSA	0	80
HSA	HSA	100	90
HSA	MeHSA	0	0
MeHSA	HSA	90*	0
MeHSA	MeHSA	30*	100

I = immediate reaction; D = delayed reaction.

*Mild edematous reactions; no petechiae seen.

tized with the corresponding methylated albumin. On the other hand, HSA was superior to MeHSA in eliciting 3 hour reactions in MeHSA-sensitized mice; 3 hour reactions in MeBSA-sensitized animals were very few, whether MeBSA or BSA constituted the test antigen. These findings suggest that the lack of 3 hour reactions in mice sensitized and tested with methylated serum albumin is due both to poor humoral antibody response and to inability of methylated albumins as test antigens to react readily with any such antibodies formed; they accord with the results of the PCA and systemic tests described earlier.

Immunodiffusion tests

Still further confirmation of these various interrelationships was obtained by immunodiffusion comparisons of precipitin reactions between native and methylated albumins and their respective antisera. As exemplified by data from Fig. 5, these tests demonstrated that methylated albumin has several antigenic determinants in common with native albumin, and antiserum to the

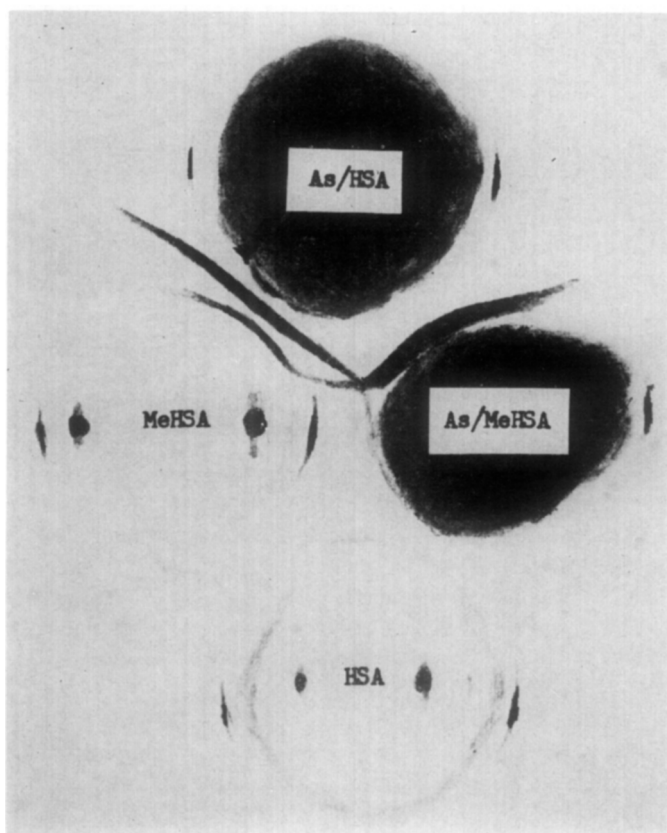


Fig. 5

Photo-drawing of immunodiffusion test in gelatin comparing the interrelationships of MeHSA and HSA through the use of their respective antisera.

former can react with these, though only weakly; that methylated albumin lacks some of the determinants found on the native albumin, and antiserum to methylated albumin lacks the corresponding antibodies; that methylated albumin has no determinants not also found on native albumin as detectable by precipitins; and that methylated albumin can precipitate heavily with antiserum to native albumin if not prevented from doing so (i.e., by having to diffuse first through a medium which complexes it, such as agar³⁰).

DISCUSSION

In response to injection of foreign native serum albumins in complete or incomplete Freund adjuvant, mice develop both Arthus and delayed hyper-

*Although there appears in Fig. 5 to have been a precipitate formed by As/MeHSA reacting with As/HSA, this precipitate actually is part of the precipitin band extending across the reaction area, and between MeHSA and As/HSA depots as well, formed by HSA diffusing under both MeHSA and As/MeHSA depots and reacting with As/HSA. The unusual precipitin band geometry probably is due to a more rapid diffusion of HSA beneath these two depots, where the gelatin gel remains wetter, than between them.

sensitivities, first and more strongly the former and second the latter.¹⁷ As has been pointed out by others,^{3, 5, 13} this situation complicates experiments on delayed hypersensitivity in mice to purified proteins. But according to results which we report here, this predicament can be avoided by using methylated serum albumins.

Mice injected subcutaneously with either MeHSA or MeBSA in incomplete Freund adjuvant preferentially develop strong delayed hypersensitivity specific for these altered and cationic proteins.* Their humoral antibody response is variable and weak and is nonspecifically directed against both native and methylated albumin. Because of the weakness of this response and because, when used for skin tests, methylated albumins probably combine rapidly with surrounding predominantly anionic tissue proteins, Arthus-type skin reactions to these antigens are either very weak or, more often, grossly undetectable. Hence with methylated albumins delayed hypersensitivity reactions to protein antigens can be studied in mice without significant interference by superimposed Arthus reactions.

Delayed hypersensitivity skin reactions similarly uncomplicated by Arthus reactions have been observed previously in mice to the purified polysaccharide dextran.¹⁸ These reactions are interesting to contrast with reactions to methylated albumins. Though macroscopically the same, microscopically the predominant infiltrating cell throughout the dextran reaction is the polymorphonucleocyte. That this is a characteristic of the interaction between mouse and polysaccharide, as we have suggested before,^{18, 31} rather than of mouse delayed hypersensitivity is supported by results reported here; for although sensitized mouse reactions to methylated albumins are heavily infiltrated by cells, monocytic cells predominate in all except the first few hours of the reaction. In this respect delayed hypersensitivity reactions in mice to methylated albumin conform to the histologic picture seen also in mice to nonpolysaccharide antigens and in guinea pigs to both proteins^{8, 32, 33} and polysaccharides³¹ when coexisting Arthus hypersensitivity is weak or absent.

Consistently, there is an early influx of polymorphonucleocytes in mouse delayed hypersensitivity reactions to methylated serum albumins; this type of influx occurs also in delayed hypersensitivity reactions of other antigens and animals.^{8, 33, 34} This would suggest that the polymorphonucleocyte is associated somehow with delayed hypersensitivity reactions, not necessarily as a cause but perhaps as an indicator of, or contributor to, early events. Since these cells often signal a humoral antibody reaction with antigen, this idea is supported indirectly by our unexpected finding that delayed hypersensitivity to methylated albumin in mice could not be transferred passively with lymphoid cells alone but required simultaneous transfer of antiserum and cells. The antiserum probably transferred enough humoral antibody to initiate,

*Gell and Benacerraf,² in their review of 1961, mention detecting delayed hypersensitivity in guinea pigs to salmine, a low molecular weight basic protein; and Green and colleagues³⁸ report detecting delayed hypersensitivity also in guinea pigs to a 50,000 molecular weight polymer of L-lysine. But no particular significance was placed on such findings by these writers. Apparently, the capacity of basic proteins or polypeptides to induce hypersensitivity has received little attention.

through the pharmacologic effects of this antibody combining with its antigen either in a very mild Arthus or anaphylactic reaction, the accumulation of enough sensitized lymphoid cells for the delayed reaction to become self-sustaining. Without such a preceding reaction, and providing the antigen is innocuous, such cells as might occasionally circulate into the test area might also pass on to other parts of the body without reacting. Thus a humoral antibody reaction, though perhaps quite weak, may be necessary to trigger a cellular antibody reaction which, once started, becomes self-perpetuating. Allergens like dextran, microbial extracts, or chlorodinitrobenzene may be sufficiently irritating themselves not to require triggering by an auxiliary humoral antibody reaction to set off their delayed hypersensitivity reactions.

This idea needs further investigation, but it agrees with the findings of Asherson and Loewi,³⁵ which were remarkably like ours; delayed hypersensitivity in guinea pigs to serum proteins, ovalbumin, or hemocyanin could be transferred only with lymphoid cells and serum, whereas similar delayed hypersensitivity to tuberculoprotein or blood group substance could be transferred with just the cells. It also may help to explain difficulties which have been encountered by some other workers³ in transferring delayed hypersensitivity passively in mice with cells alone. We reported that such could be effected,¹⁶ but in those early experiments we transferred the cells 48 hours before skin-testing the recipients. This could have allowed time for the transferred cells to make enough humoral antibody for the necessary triggering reactions. Unless antibody were already present, in experiments such as reported here in which skin tests are performed at the time of transfer, too little antigen might remain at the test site to react with humoral antibodies eventually made by the transferred cells to trigger a cellular antibody reaction.

Much has been learned about the comparative natures of the antigenic sites reactive in guinea pig immediate and delayed hypersensitivities because ways were found to make these animals respond selectively to various haptens and hapten carriers.⁸ Now, similar data should become available for these hypersensitivities in mice. This will be evident from the following discussion.

Methylation changes serum albumins from strongly anionic to strongly cationic, and this change probably more than any other accounts for the methylated antigens' different immunologic properties. Methylated albumin combines strongly with such anions as agar, so much so that immunodiffusion tests required that we substitute gelatin for either agar or agarose in order to detect precipitins reactive with this albumin.³⁰ These tests indicated that methylation had blocked or inactivated some antigenic determinants found on native albumin but had left most intact, and it had created no new ones insofar as humoral antibody response could reveal. But in sharp contrast to this, delayed hypersensitivity to methylated serum albumins was specific for these albumins themselves. There are several interesting implications to this last point.

It appears to be the first evidence available that delayed and immediate

hypersensitivities in the mouse, like the guinea pig,⁸ can be directed against different aspects of a single antigen. It provides independent definitive separation of immediate and delayed hypersensitivities in mice to protein antigens. It indicates that the triggering of a passively transferred delayed hypersensitivity reaction by antigen reacting with humoral antibody, as described previously in this article, is not antigenically specific; the same effect should be obtained by allowing any other antigen-humoral antibody system to react at an appropriate time in a skin-test site intended eventually to develop a delayed hypersensitivity reaction.

Why should an antigen with no new determinants induce a delayed hypersensitivity not detectable by determinants found on the native antigen? First, methylation may have changed it physically or chemically so that it did have new determinants as detected by delayed hypersensitivity antibodies even though not by humoral antibodies. This explanation is reasonable speculation according to current knowledge of the usual differences between determinants reacting respectively with cellular and humoral antibodies. For instance, a guinea pig injected appropriately with a foreign protein altered by addition of some chemical hapten will form humoral antibodies against the hapten and cellular antibodies against the protein.^{2, 8} Therefore, a different kind of protein with the same hapten attached will elicit a humoral antibody reaction; although these two proteins may be quite different, if humoral antibodies are directed only against the hapten, humoral antibody reactivity would reveal no difference between the two antigens. On the other hand, since delayed hypersensitivity antibodies are directed primarily against portions of the carrier antigen rather than the attached hapten, delayed hypersensitivity toward the first carrier will be specific and quite distinct from that toward the second, and the second protein carrier would not elicit a delayed hypersensitivity reaction. Methylation very well could be inducing changes in broader portions of serum albumins than are the determinants eliciting humoral antibody formation.

A second explanation, perhaps more likely without necessarily conflicting with the first, is that because of its new physicochemical characteristics methylated albumin combines rapidly with native tissue proteins forming a "self + X" complex³⁶ which, to the experimental animal, constitutes a homograft and elicits a delayed hypersensitivity response. This is thought to account for the allergenicity of simple chemicals^{2, 37} and other more complex antigens,^{2, 32} and it would be compatible with perservation of mediocre humoral antibody reactivity to most of the determinants present on the native albumin. These, though intact, might be so blocked by the attached host proteins as to constitute only weak antigenic stimuli.

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