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Inactivation of the Skin-Sensitizing Antibodies of Human Allergy by Thiols. (27690)

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The skin-sensitizing antibody or reagin is considered to be an important pathogenetic factor in human atopic allergy. Although many of the unique characteristics of this antibody are well known, a clear understanding of its physicochemical properties has been impeded by discordant findings(1). One method which has contributed valuable insights into antibody structure and classification is *in vitro* exposure to sulfhydryl (SH) compounds (2). This treatment inactivates high molecular weight (19S) antibodies(3), dissociating them into 7S subunits, presumably through reductive cleavage of disulfide bridges(2). Ordinary 7S (γ_2-globulin) antibodies are not affected(3) unless a denaturing agent is used in conjunction with SH treatment(4). Since the effect of SH compounds on the activity of human reagins was unknown, this investigation was undertaken. Data will be presented to show that *in vitro* SH treatment of the reagin-containing sera of allergic patients destroys the skin-sensitizing activity of these sera.

Materials and methods. After screening donors for a history of hepatitis, Wassermann negative sera from 16 allergic children and adults were selected for their ability to passively sensitize non-atopic human skin to subsequent challenge with the appropriate allergen(s) (the Prausnitz-Küstner or P-K reaction). The sera were sterilized by Seitz or Millipore filtration and stored at 4°C. The reagins represented included those to pollens, animal danders, molds and foods. The thiols employed in the majority of experiments were β-mercaptoethanol (ME) and DL-penicillamine; L-cysteine and β-mercaptoethylamine (MEA) were also used in certain later studies.

In a typical experiment, to each allergic serum diluted 1:3 with triethanolamine buffered saline (TBS) (pH 7.35, $\mu = 0.15$) was added an equal volume of 0.2M thiol which had been freshly prepared in TBS and adjusted, when necessary, to pH 7.2-7.3. Thus, the final thiol concentration was 0.1M and the final serum dilution was 1:6, giving a protein concentration of approximately 1%. Untreated controls consisted of aliquots of the same sera diluted equally with TBS alone and thereafter handled identically to the thiol-treated aliquots. Incubation in sterile, closed tubes was usually at room temperature for 24-48 hours in experiments with ME or MEA, and at 37°C for 24 hours with penicillamine or cysteine. After incubation, treated and control sera were dialyzed (separately) for 36-48 hours at 4°C against TBS with sufficient changes to reduce the calculated thiol concentration to 10^{-8}M or less. The dialyzed sera were then sterilized by Millipore or Seitz filtration. After 48 hours were allowed for bacterial cultures, bioassays were begun.

Antibody activity in the control and treated sera was assayed, usually without further dilution of the sera, by the P-K reaction in normal, non-allergic human volunteers unreactive in direct intradermal skin tests with the allergens concerned. Each treated serum and its untreated control were uniformly

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TABLE I. Effect of Mercaptoethanol on P-K Reactions of Human Reagins.

Allergic serum (1:6)	Allergen*	Mean wheal diameters (mm) given by passively transferred sera: Tested on back, Untreated	Tested on back, Treated†	Tested on back, Untreated	Tested on back, Treated†
Ba.	Plantain	6.5	—‡	6.5	—
	Bermuda grass	8.5	—	6.0	—
Br.	Ragweed	15.5	9.0	17.0	3.5
En.	Ragweed	11.0	—	N.D.	N.D
	June grass	9.5	—	"	"
Li.	Ragweed	12.5	—	10.5	—
Mi.	Ragweed	6.5	—	8.5	—
Wa.	Alternaria	15.5	5.0	12.5	—
	Orchard grass	12.0	—	12.0	—
	Ragweed	7.0	—	8.5	—
Wi.	Sweet vernal	14.0	—	16.5	—
	Ragweed	15.5	2.5	15.0	—

* All allergen extracts used contained 1000 P.N.U./ml except alternaria extract which contained 0.01 mg total nitrogen/ml.

† 0.1 M mercaptoethanol for 34 hr at room temperature.

‡ (—) indicates either no reaction or a reaction no greater than that given by the allergen alone in an unsensitized site.

N.D. = Not done.

tested in 2 replicate intradermal sites on the back and upper arms. To minimize the effect of variable reactivity of different areas of skin, a given treated serum and its control were paired and injected either into adjacent sites (at least 4 cm apart) or, less frequently, into corresponding sites on the left and right sides of the body. The position of a given treated-control pair was random and well separated from the replicates of this pair. In several earlier experiments assay of the same sera in 2 or 3 recipients confirmed the reproducibility of the reactions. Thereafter, a single recipient usually served for each experiment. The sensitizing injections consisted of 0.1 ml in each site. Challenge of these sites was made 48 hours later with 0.01-0.02 ml of the specific allergen. Adjacent unsensitized skin sites were tested with the allergens alone at the same time, as a direct control. At the height of the reactions, approximately 20 minutes after challenge, the longest and shortest diameters of the resulting wheals were measured (in mm), and their arithmetic means recorded. The allergens were extracts currently in use in the Allergy Clinic, Children's Hospital of D.C., standardized by Protein Nitrogen Units (PNU) or Total Nitrogen and, in general, were employed in the usual concentration used in skin tests of allergic patients.

Incomplete anti-Rh_o antibodies and pathologic cold hemagglutinins were measured as previously described(5). Paper electrophoresis and densitometric analysis were carried out in the Spinco model R system (procedure B).

Results. The effect of thiols on the reagins of the 16 allergic sera were studied in 15 recipients in 10 separate experiments. A representative portion of the results is presented in Tables I, II, and III, each table representing a single experiment using one recipient. From each table it is evident that P-K activity was either completely lost or sharply reduced by treatment with mercaptoethanol (ME), penicillamine, or cysteine. Similar results were consistently obtained with these thiols in other experiments with these same sera and with the other allergic sera not shown. Mercaptoethylamine (MEA), on the basis of more limited studies with 6 sera, appeared to be the least effective of the 4 thiols at physiologic pH,§ although a definite effect

§ While present study was in progress, we learned that Gyenes and Sehon(6) have recently observed loss of skin-sensitizing activity after treatment of allergic sera with 0.1M MEA at pH 8.2.

TABLE II. Effect of Penicillamine on P-K Reactions of Human Reagins.

Allergic serum (1:6)	Allergen*	Mean wheal diameters (mm) given by passively transferred sera: Tested on back, Untreated	Tested on back, Treated†	Tested on upper arm, Untreated	Tested on upper arm, Treated†
Ba.	Bermuda grass	9.5	—‡	10.5	—
	Plantain	10.0	—	10.0	—
En.	June grass	8.0	—	9.5	—
	Ragweed	10.5	3.0	11.5	—
Hi.	Orchard grass	7.5	—	7.5	—
Li.	Ragweed	11.0	—	10.5	3.5
Mi.	Ragweed	6.5	—	9.0	—
	Timothy	6.0	—	10.0	—
Pr.	Orchard grass	10.0	—	9.5	3.5
Tu.	Ragweed	12.0	4.0	12.5	6.5
	Dog	9.0	3.5	8.0	—
Wig.	Elm	11.0	—	9.0	—
	Red top	8.0	—	6.5	—
Wil.	Ragweed	11.5	4.0	11.0	—
	Sweet vernal	11.5	—	11.5	5.5

* All allergen extracts used contained 1000 P.N.U./ml.
† 0.1 M penicillamine for 24 hr at 37°C.
‡ (—) indicates either no reaction or a reaction no greater than that given by the allergen alone in an unsensitized site.

was observed in each serum. In all experiments the untreated control sera retained full activity; *i.e.*, it was repeatedly found that there was no detectable difference between the activity of a control serum, carried through all operations except addition of the thiol, and that of the original serum freshly diluted 1:6 at the time of testing. In one experiment SH treatment was carried out by dialyzing reaginic sera against 0.1M ME; the results were similar to those obtained by direct addition of ME.

TABLE III. Effect of Cysteine on P-K Reactions of Human Reagins.

Allergic serum (1:6)	Allergen*	Skin site tested	Mean wheal diameters (mm) given by passively transferred sera: Untreated	Treated†
En.	June grass	Back	10.5	4.0
		Arm	8.5	—‡
Ha.	Ragweed	Back	14.0	—
		Arm	10.0	—
Mi.	"	Back	7.0	—
		Arm	6.5	—
Pr.	Orchard grass	Back	11.0	—
		Arm	6.0	—
Tu.	Ragweed	Back	10.5	—
		Arm	9.0	—

* All allergen extracts used contained 1000 P.N.U./ml.
† 0.1 M cysteine for 24 hr at 37°C.
‡ (—) indicates either no reaction or a reaction no greater than that given by the allergen alone in an unsensitized site.

Studies of minimal incubation time with 0.1M ME showed that at room temperature inactivation of reagins was complete with all sera in 48 hours, with all but the highest titered sera in 24-36 hours, and with some sera in 12 hours; at 37°C, incubation could be shortened to 30-90 minutes. Penicillamine had very little effect on reagins at room temperature. Cysteine was used only at 37°C, under a nitrogen atmosphere to retard the formation of insoluble crystals, presumably cystine. Three sera, Br., Tu., and another not shown, had the highest P-K titers (approximately 1:1000 against ragweed) by serial 2-fold dilution. After treatment with ME at room temperature for *less than 48 hours* or with the other thiols in the usual way (see *Methods*), these 3 sera often retained considerable activity (*e.g.*, Tables I and II). In such instances, P-K tests with serial 2-fold dilutions of the treated and control sera

TABLE IV. Sulfhydryl Treatment of Incomplete Anti-Rh and Pathologic Cold Hemagglutinins.

Serum	Type of antibody	Treatment	Reciprocal of titer*
Ro.	Incomplete anti-Rh	Control	48
		ME†	48
Co.	*Idem*	Control	48
		ME	48
Ro.	"	Control	48
		Pen.†	48
Co.	"	Control	48
		Pen.	48
Pe.	Cold agglutinin	Control	5120
		ME	Neg.
Pe.	*Idem*	Control	10,240
		Pen.	Neg.

* The initial dilution for anti-Rh titer was 1:6; for cold agglutinin titer, 1:80.

† ME = mercaptoethanol, 0.1 M, 34 hr at room temperature; Pen. = penicillamine, 0.1 M, 24 hr at 37°C.

readily demonstrated a 3 to 5 tube reduction in titer after SH treatment. Thus, the degree of inactivation of a given allergic serum appeared to depend upon its original reagin titer, activity of the thiol, and length of incubation.

To demonstrate (a) that the effect of our particular thiol preparations on other types of human antibodies was in accord with previous observations, and (b) that the experimental conditions were not bringing about a general denaturation of serum antibodies, 2 sera containing incomplete anti-Rh_o (anti-D) antibodies and one serum containing pathologic cold (anti-I) hemagglutinins were treated identically to and simultaneously with the allergic sera in several experiments using ME and penicillamine. As expected(3), the incomplete anti-Rh antibodies, known 7S (γ_2-) globulins(3,7) were unaffected by SH treatment (Table IV). The cold agglutinins, known 19S γ_1M- (β_2M-) globulins(3,8), were readily inactivated (Table IV), and appeared to be more SH-sensitive than reagins in that inactivation of these particular cold agglutinins by ME was more rapid and could be accomplished with penicillamine at room temperature. The limitations of basing a conclusion on a single 19S antibody and of comparing results of 2 such dissimilar assays are recognized, however. As a further control, no significant difference in serial 2-fold titers of tetanus antitoxin‖ in 3 ME-treated allergic sera and their controls was found by Dr. R. A. Finkelstein, using bioassay in mice.

Early in the course of these studies it was noted that most of the allergic sera, although sterile, became turbid during incubation with ME. The degree of turbidity varied greatly from serum to serum despite identical conditions, although all sera became turbid if incubation were sufficiently extended. At room temperature slight turbidity was first detected at 14-18 hours of incubation and thereafter steadily increased so that, after several days, a gelatinous or lacy precipitate resulted. With ME at 37°C, turbidity was apparent in one hour and maximal in 8 hours. Normal sera were equally susceptible to these changes, again some sera more than others. The greater the protein concentration, the more intense and more rapid was turbidity formation; in fact, undiluted serum quickly gelled. Using buffered diluents other than TBS, but similar in pH and ionic strength, did not alter turbidity formation. By actual experiment the curious variation from one serum to another could not be correlated with duration of storage, timing of venapuncture relative to meals, or content of free hemoglobin.

Since no mention of this turbidity formation could be found in many previous references to ME treatment of human serum under comparable conditions of pH, protein concentration and ME concentration, a large volume of ME was fractionally redistilled on a spinning band column, stored in small ampoules under nitrogen at –20°C, and used for all subsequent work with this thiol. Despite a slight correction in its refractive index, this redistilled ME still produced turbidity. Moreover, similar turbidity was produced by MEA, and, to a lesser extent, by cysteine. With penicillamine, turbidity was not generally observed unless the usual 24-hour incubation was exceeded or a higher than 1% protein concentration employed.

Of greatest relevance to the subject of this report are the observations (a) that the for-

‖ Tetanus antibody, as measured by hemagglutination, has been found in 7S fractions of human serum(9).

mation of turbidity did not appear to be a necessary accompaniment of reagin inactivation by thiols, and (b) that neither anti-Rh antibodies nor tetanus antitoxins were measurably affected by the development of rather marked turbidity in these sera during SH treatment. Penicillamine regularly gave clear inactivation of reagins (Table II) without causing turbidity. Moreover, by selecting those allergic sera with the least tendency to turbidity and by utilizing the data on minimal incubation time, it was repeatedly possible, at both room temperature and 37°C, to destroy all reagin activity with ME before turbidity appeared. Finally, if a greater dilution (*e.g.*, 1:40 or more) of the higher-titered sera were incubated with ME, inactivation could be demonstrated in the absence of turbidity.

To gain some idea of what proteins were participating in the development of turbidity, 4 sera made minimally to markedly turbid with ME were clarified as much as possible by centrifugation at 27,000 × *g*. Comparison of the paper electrophoretic patterns of these supernatants to "supernatants" from untreated aliquots of these sera revealed reductions in albumin, in the main β-globulin peak, and, less consistently, in the α_2-globulin peak, with an increase in the β_2-γ_1 area probably representing, at least in part, turbid material which could not be spun down. The α_1- and main γ-globulin peaks were essentially unchanged. Supernatants from 2 sera made mildly turbid with penicillamine (using 3% protein concentration) gave similar results except for normal albumin.

Discussion. The foregoing observations indicate that human reagins lose their *in vivo* biological activity during treatment with sulfhydryl (SH) reducing agents under conditions (a) in which known 7S human antibodies, γ_2-globulins, retain full *in vitro* biological activity and (b) in which the *in vitro* activity of known 19S human antibodies, γ_1M-globulins, is destroyed.

The mechanism of SH inactivation of reagins remains to be clarified. Since SH compounds apparently act upon proteins through a specific effect on disulfide bonds(10,11), the efficacy of 4 different thiols strongly suggests that reductive cleavage of one or more disulfide bonds in the reagin molecule plays a primary role in its inactivation. Conversely, on this basis it seems likely that reagins, like the 19S γ_1M-globulins but unlike ordinary 7S γ_2-globulins, contain one or more disulfide linkages which are both accessible to direct SH reduction and critical for biological activity. It does not necessarily follow, however, that inactivation of reagins involves dissociation into smaller fragments in the manner of 19S globulins. Ishizaka and associates(12) have recently reported that the *in vivo* skin sensitizing function of rabbit γ-globulin (as antibody) and human γ-globulin (as antigen) is destroyed by SH treatment (plus iodoacetate) while their participation in reversed PCA, precipitin, or complement fixation reactions is spared. Although all of these *in vivo* and *in vitro* antibody activities may not necessarily be mediated by the same γ-globulin molecule, it seems quite possible, as suggested by Ishizaka, that SH reduction may affect the tissue binding function of an antibody with little or no effect on its antibody combining sites. The work of Ovary and Karush(13) is compatible with this concept. Since assay of human reagins is uniquely dependent upon their ability to sensitize human skin, their inactivation by thiols could result solely from an interference with their tissue binding mechanism. It is also possible that SH compounds act upon some other functional aspect of reagins required for their full biological expression, *e.g.*, the hypothesized lipoprotein cofactor which may participate in the P-K reaction(14). On the supposition that such a cofactor, rather than reagin itself, could be the thiol-sensitive element in allergic sera, a single experiment was done to determine whether 2 volumes of normal serum could restore P-K activity to thiol-inactivated allergic sera. No restoration was observed.

Progressive increases in viscosity and turbidity in solutions of purified proteins, *e.g.*, bovine albumin, ovalbumin, and bovine ribonuclease, during reduction with thiols has been observed in several laboratories(15,16) and ascribed to the transformation of intramolecular disulfide bonds to intermolecular disulfide linkages with resulting aggregation of protein molecules (see also(17)). This would seem to be the best available explanation of

the turbidity observed in our SH treated sera, even though the thiol was present in excess. Only further study can determine whether inactivation of reagins involves such intermolecular aggregation.

Very recent work(18,19) indicates that human reagins belong to a special class of 7S immune globulins, the γ_1A-(β_2A-) globulins, in keeping with their many exceptional properties. To elucidate the mechanism by which thiols affect reagins, work is now in progress to determine the effect of SH compounds on the physicochemical nature of γ_1A-globulins as a class. This and future studies on other antibody activities in the γ_1A-globulin class will test our present impression that reagins, and possibly, therefore, γ_1A-globulins as a class, may be intermediate in SH sensitivity between γ_2-globulins and γ_1M-globulins.

Whether SH compounds disrupt the structure of reagins or merely affect their tissue-sensitizing mechanism, it is conceivable that thiols may have *in vivo* usefulness in certain allergic patients refractory to other measures. While much remains to be learned about *in vivo* effects of SH drugs, considerable clinical experience with D-penicillamine(20) suggests that this thiol is well tolerated. A preliminary study to determine whether D-penicillamine is effective against reagins *in vivo* is planned.

Summary. *In vitro* exposure of 16 sera from patients with atopic allergy to several different sulfhydryl compounds resulted in inactivation of their skin-sensitizing (reagin) activity, as measured by passive transfer to normal human skin (Prausnitz-Küstner reaction).

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