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ANTIGENIC RELATIONSHIPS IN ATOPY

I. Immunoprecipitation of Different Atopic Allergens and Synthetic Model Compounds with Antisera to Human Dandruff Allergen.

By

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INTRODUCTION

One very striking aspect of the syndrome of atopy in man is the multiple sensitivity to a variety of apparently unrelated allergenic materials. A high incidence of cross-reactivity for totally different allergens has repeatedly been observed during intradermal assay in atopic subjects manifesting clinical sensitivity to one of these allergens only. Of the several explanations suggesting themselves for this phenomenon, -common antigens in diverse allergen preparations, polyvalent sensitization—the proposal of shared antigenic (or allergenic) determinants among otherwise distinct (glyco) protein molecules has gained attractiveness since the demonstration of N-glycosidic lysine-sugar units within the molecular framework of several atopic allergens (4, 7, 8, 10, 14, 16).

It was considered that the possible antigenic relationship between different allergens in this sense of shared determinants, structurally identical or closely related, might be detectable by studying the *in vitro* interaction of different allergens with an antiserum to a single allergen preparation. The present paper reports precipitation reactions observed during immunodiffusion in systems of rabbit antiserum to purified human dandruff allergen, with different allergens or synthetic protein-sugar model conjugates as the antigens.

MATERIALS AND METHODS

Antisera to purified human dandruff allergen, fraction E (12), were raised in two rabbits as described elsewhere (6); the antisera were pooled because no significant qualitative or quantitative differences were detected by conventional serological techniques. A full description of the dandruff allergen E and of its reaction with homologous rabbit antiserum is being published elsewhere (6). Analytical techniques involved gel-diffusion methods in agar gel at pH 8.2 (veronal buffer, incorporating merthiolate 1:100 000) on microscope slides according to *Ouchterlony*; diffusion was allowed to proceed in a moist chamber at room temperature for 24-48 h. Immuno-electrophoresis was done in agar gel at pH 8.6 by the *Scheidegger* microtechnique (24). The pooled immune serum was used undiluted; 5 per cent solutions of the antigens or allergens in saline or in veronal buffer pH 8.6 were applied unless otherwise stated. A description of the nature and properties of the atopic allergens and synthetic protein-sugar model compounds has been given in separate communications (5, 7, 8, 10, 17).

RESULTS

The composition of the skin-reactive allergen fraction E from normal human dandruff has been investigated by chemical (12) and immunochemical (6) methods. The allergen fraction, used for raising the rabbit antiserum, proved to be very heterogenous, and the occurrence of human serum albumin, 2.5 S- α_2 -glycoproteins, epidermal glycoproteins and, presumably, of prekeratins or keratin-derivatives has been established. Immunochemically, no other serum proteins were detected; with the aid of specific antisera the absence, in particular, was ascertained of α_2 -macroglobulin, α_1 -acid glycoprotein, α_1 -antitrypsin, β -lipoprotein, β_2 -glycoprotein, transferrin, IgA, IgG and IgM.

The earlier chemical evidence for N-glycosidically bound sugar in the protein constituents of the dandruff allergen (7, 8, 10, 12) could be extended immunochemically by the application of synthetic conjugates of D-ribose and serum albumin or 2.5 S- α_2 -glycoproteins for shifting the precipitin arcs in the immunoelectropherograms to the position occupied by these -apparently modified- serum proteins in the pattern obtained with dandruff allergen E and its antiserum (6). Sev-

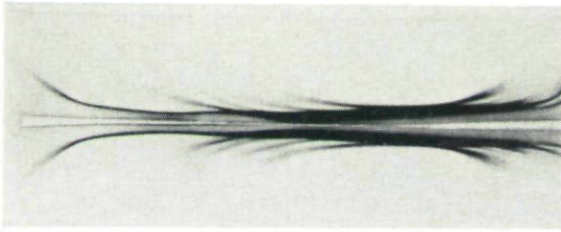


Fig. 1.

Immunoelectrophoresis of human dandruff allergen, fraction E, against homologous rabbit antiserum; agar-gel electrophoresis of dandruff fraction E, 5 per cent solution in veronalbuffer pH 8.6 (top hole and bottom hole, anode on the right), immunodiffusion against undiluted antiserum in centre groove.

Stain: Naphtol Blue Black.

eral of the other constituents in the allergen fraction were found to share antigenic determinants.

The complex composition of the dandruff allergen fraction E and the multiplicity of precipitating antibodies in the homologous antiserum becomes apparent in the immunoelectrophoretic pattern reproduced in Fig. 1.

Though recognizing the restrictions imposed on conclusions drawn from experiments with this antiserum, it was decided to study its reaction with other atopic allergens in order to uncover possible antigenic relationships to the components in the dandruff allergen fraction.

a. *House Dust Allergen*

It does not surprise that cross-precipitation readily occurred with both the D- and E-fraction of house dust (Fig. 2). Several reports have communicated the presence of human dandruff components in the active allergen fractions from house dust (11, 22, 23). The extensive cross-precipitation of the D-fraction of house dust, in particular, adds significantly to the earlier clinical (13), chemical (12) and immunochemical (11) evidence for dandruff constituents and serum proteins in this fraction. The D-fraction of house dust, for instance, shares full antigens (serum albumin) with the human dandruff frac-

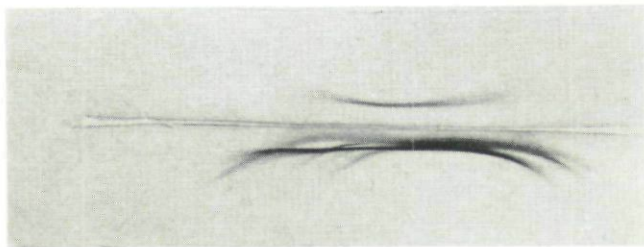


Fig. 2.

Immunoelectrophoresis in agar gel of house dust fractions E and D against rabbit anti human dandruff (fraction E) serum; electrophoresis: 5 per cent solutions of dust fraction E (top hole) and of dust fraction D (bottom hole) at pH 8.6, anode on the right. Undiluted antiserum for immunodiffusion in centre groove.

tion E (11, 22, 23); however, the relationship with respect to the very potent E-fraction of house dust is less evident, because this allergen—though not free from traces of albumin and of minor dandruff constituents (21)—is predominantly composed of plant glycoproteins (13, 21). The single precipitin arc observed with dust fraction E (Fig. 2) is not in the position of albumin and, consequently, there is room here for suggesting cross-precipitating to be due to shared structural determinants among the chemically distinct allergen fractions E from house dust and from human dandruff.

b. *Horse Dandruff Extract*

The antiserum for human dandruff fraction E precipitated the non-dialyzable portion and a partially purified allergen fraction from horse dandruff. The single precipitin arc observed, in the position of an α_2 -globulin, could not be ascribed to the trace amounts of horse serum α_2 -macroglobulin reported in horse dandruff extract (25), nor to possible traces of native horse serum 2.5S- α_2 -glycoproteins. Further immunochemical analysis has led to the proposal that horse dandruff and human dandruff low-molecular weight α_2 -glycoproteins share determinants which are absent from the native preparations isolated from the serum (6).

c. Other Atopic Allergens

Several purified allergen samples were screened by the method of double diffusion in agar gel for possible precipitation reactions with the anti human dandruff serum. With the exception of the purified mould allergen *trichophytin* (1) all allergens investigated gave rise to one or more precipitin lines in the immunoprecipitation pattern. However, in a few cases these precipitin lines were too weak for photographic recording, while the low antibody titre for several other allergens necessitated a refill of the antigen wells after 24 h of diffusion and an extension of the diffusion period to 48 or 72 h. Some representative patterns have been collected in Fig. 3.

Fig. 3 a shows the formation of 4 precipitation lines of the anti dandruff serum with the non-dialyzable components from an allergenic extract of aged chicken *feathers*. One of the lines nearest the antigen well completely fuses with the single precipitin line obtained with the allergen fraction F from *Succus Liquiritiae* (2).

Fig. 3 b gives the precipitation pattern obtained with the allergenic feather extract and the purified *kapok* fraction E (4). Of the three precipitation lines observed with the feather extract, one exhibits antigenic identity to one of the kapok antigens.

Precipitation reactions of the anti dandruff serum with a purified allergenic extract of *hay* (fraction HH-C, compare ref. 15), and with *house dust allergen* fraction E and homologous *dandruff allergen* E are shown in Fig. 3 c. The precipitin lines with HH-C were only very weak; however, examination with a photographic enlarger indicated reactions of identity between individual components of the three allergen preparations.

A similar conclusion of antigenic similarity was reached for constituent substances of several other allergen preparations. Fig. 3 d shows cross-precipitation of the anti dandruff serum with allergenic fractions from *tomato*, *feathers*, *hay*, *kapok*

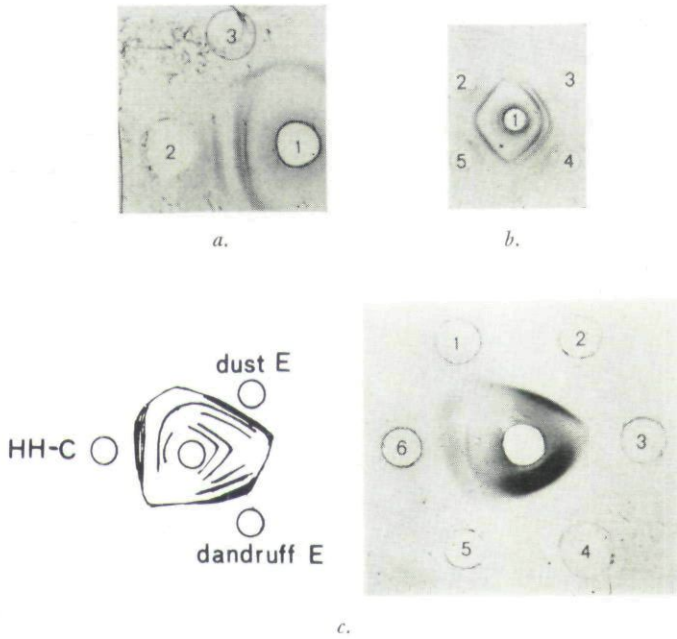
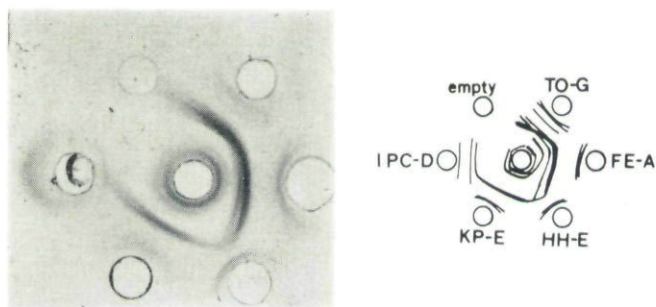


Fig. 3.

- a. Ouchterlony gel diffusion pattern of anti human dandruff serum ($30\ \mu\text{l}$, undiluted, well no 1) against dialyzed feather extract FE-A ($30\ \mu\text{l}$ 5 per cent solution, well no 2)) and allergen fraction SL-F from liquorice ($30\ \mu\text{l}$ 5 per cent solution, well no 3).
- b. Diffusion pattern of anti human dandruff serum (well no 1) against kapok fraction KP-E (wells 2 and 5) and feather extract FE-A (wells 3 and 4); antigen wells filled with $30\ \mu\text{l}$ 5 per cent solutions.
- c. Precipitation pattern during immunodiffusion of house dust allergen fraction E (well no 2, $30\ \mu\text{l}$ 5 per cent solution), homologous human dandruff fraction E (well no 4, $30\ \mu\text{l}$ 5 per cent solution) and hay allergen fraction HH-C (well no 6, $30\ \mu\text{l}$ 5 per cent solution) against undiluted anti human dandruff (fraction E) serum (centre well, $30\ \mu\text{l}$). Wells 1, 3 and 5 empty.

and *ipecac*, while the fusion of several of these precipitin arcs is indicative of reactions of identity. The fusion of two of the precipitation lines of feather extract with two of the arcs observed with the active tomato allergen fraction TO-G (16) is especially interesting, firstly because determinants are also shared with the kapok and *ipecac* allergens, and secondly be-



d.

Fig. 3.

- d. Gel diffusion of undiluted anti human dandruff serum (centre well, 30 μ l) against purified allergen fractions from tomato (TO-G), feathers (FE-A), hay (HH-E), kapok (KP-E) and ipecac (IPC-D). All fillings consisted of 25 μ l 5 per cent solutions, one refill after 24 h. Positions of separate allergens indicated in tracing.

cause these cross-reactions with the antiserum can clearly not be attributed to a common contaminant substance (for instance a product of moulds). The glycoprotein allergen from tomatoes originates in the inner parts of the fruit during non-enzymatic browning processes (16), and there is no gross chemical relationship with the keratin breakdown products in extracts of aged feathers (9).

d. Model Compounds

In view of the evidence for the incorporation of N-glycosidically bound sugar in the human dandruff allergen (6, 7, 8, 10, 12), it was considered that the cross-precipitations observed might be due to antibodies for the lysyl-sugar hapten; similar lysine-(ϵ -amino)-sugar residues have been demonstrated in other atopic allergens (4, 7, 8, 10, 12, 16, 17). Accordingly, a number of model compounds was prepared, composed of crystalline proteins complexed with sugar by coupling different monosaccharides to the ϵ -amino groups of lysine residues in the polypeptide chain; in the present study ovalbumin was used which had been made to react with

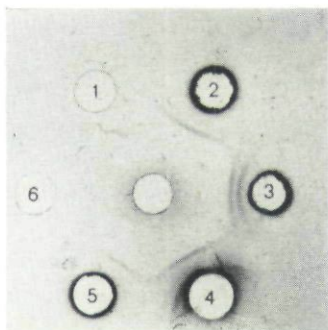


Fig. 4.

Precipitation pattern of undiluted anti human dandruff (fraction E) serum (centre well) against synthetic β -lactoglobulin-lactose complexes of different degree of conjugation. All outer wells filled with 25 μ l 5 per cent solutions, one refill after 24 h. Well designations:

1: cryst. β -lactoglobulin (control), "Available lysine" 11.3 per cent.

2: conjugate A, "available lysine" 8.3 per cent.

3: conjugate B, "available lysine" 6.7 per cent.

4: conjugate C, "available lysine" 4.6 per cent.

5: conjugate D, "available lysine" 4.4 per cent.

Well no 6 empty; total diffusion period: 48 h. For conjugates compare ref. 17.

different sugar partners, while β -lactoglobulin-lactose complexes (BLG-lact.) of different degree of conjugation were also used (17).

Fig. 4 reproduces the pattern obtained after double diffusion of the anti dandruff serum against several BLG-lact. conjugates (preparations A, B, C and D, isolated after 48, 96, 144 and 168 h of reaction with lactose respectively, compare ref. 17), representing compounds of increasing degree of conjugation (*i.e.* decreasing content of "available" lysine). The native parent protein did not react in this test; the precipitating capacity of the protein-sugar complex diminished with increasing degree of conjugation, best results (and doubling of the precipitin line) being obtained with the B-preparation (Fig. 4). These precipitation reactions could be almost completely suppressed by the incorporation of the low-molecular weight hapten N-(1-deoxy-1-D-fructosyl)- ϵ -aminocaproic acid (EACAF, ref. 5) in the antiserum prior to diffusion; the control compound ϵ -aminocaproic acid proved a far less effective inhibitor.

These β -lactoglobulin-lactose preparations differ from the parent protein by the incorporation of 1-deoxy-2-ketoses

attached to the ϵ -amino group of lysine residues, *i.e.* the glucose moiety of lactose binds to the protein amino group, while the galactose moiety is subject to elimination as a result of rearrangements in the sugar partner (17). In consequence, the above experiment proves the anti dandruff allergen serum to encompass antibody directed against the lysine-sugar determinant.

Fig. 4 also indicates that

- a. degradation reactions in the carbohydrate moiety of the protein-sugar complex—promoted by long-term incubation at 50° during the preparation of the conjugate—tend to destroy the determinant (Fig. 4).
- b. the engagement of an increasing number of lysine residues, up to a limiting value, gives rise to the appearance of a second precipitin arc in the gel-diffusion pattern. Tentatively, this may be interpreted as indicating the anti-lysine-sugar antibodies in the antiserum to be heterogeneous with respect to the chemical composition of the particular peptide portion carrying the lysine-sugar determinant; this would support the proposal that the amino acid sequence adjacent to this determinant cooperates in determining antigenic specificity (3, 4).

The possibility was considered that the specificity of the lysine-sugar determinant might still be dictated by the nature of the sugar partner. In a series of studies precipitation reactions were accordingly investigated of the anti dandruff serum with protein-sugar models prepared by the reaction of crystalline ovalbumin with glycolaldehyde, glyceraldehyde, erythrose, ribose, arabinose, fucose, glucose and lactose. With the exception of the first three products, all conjugates produced one (weak) precipitin line with the antiserum, which mutually joined in a reaction of identity (Fig. 5); a second, separate precipitin arc was seen with the ovalbumin-arabinose complex.

Accordingly, the specificity of the precipitating antibody does not depend on the nature of the sugar partner attached

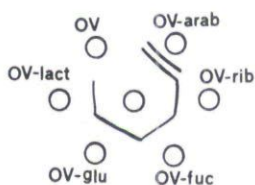
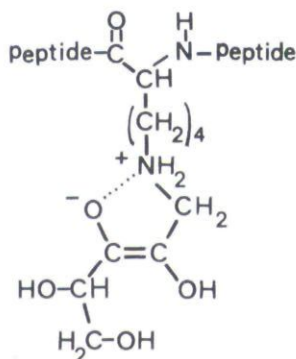


Fig. 5.

Tracing of precipitation pattern of anti human dandruff serum (centre well) diffused against synthetic ovalbumin-sugar conjugates in the outer wells, *viz.* (clockwise): cryst. ovalbumin control (OV), and further ovalbumin conjugated with arabinose, ribose, fucose, glucose and lactose respectively. Wells filled with 25 μ l 5 per cent solution, one refill after 24 h.

Total diffusion period 48 h.

to the antigen-lysine residues, but since the lower-membered aldoses did not react, and because the ovalbumin-arabinose conjugate proved the better antigen of the series, it was inferred that the antiserum largely encompassed antibody for the stabilized form of one of the enolic tautomers of the N-glycosidically bound sugar residue (5, 7, 8). Formation of this so-called "pseudo-isoxazoline" structure of the antigenic determinant (5) would, in fact, be facilitated by the configuration of the hydroxyls at C-2 and C-3 of arabinose:



At this point the question arises whether thermal denaturation of the sugar partner during the preparation of these conjugates might not have uncovered perfectly natural "hidden" determinants structurally related to similar determinants in the human dandruff allergen (for instance oligosaccharide-

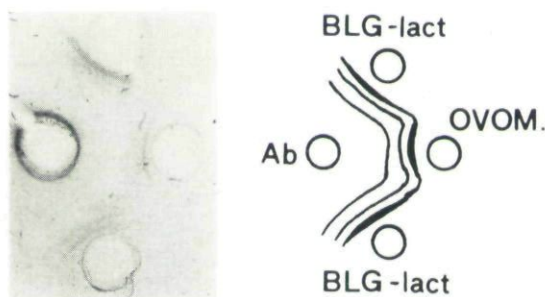


Fig. 6.

Immunodiffusion in agar gel (pH 8.2) of BLG-lactose conjugate preparation C (compare Fig. 4 and ref. 17) and ovomucoid against rabbit anti human dandruff (fraction E) serum. Well designations in tracing correspond to those in original.

Antigen wells filled with 25 μ l 5 per cent solutions, one refill after 24 h.

Total diffusion period 48 h.

sialic acid chains). A subsequent experiment made this unlikely: the anti dandruff serum precipitated the crystalline egg-protein ovomucoid, and the Ouchterlony gel plate revealed antigenic identity with the β -lactoglobulin-lactose conjugate (Fig. 6). Native β -lactoglobulin, ovalbumin, conalbumin or lysozyme did not precipitate the anti dandruff serum. Unfortunately, extensive studies have hitherto failed to disclose N-glycosidically bound sugar in ovomucoid; yet, this protein proved to be extremely, and specifically, potent during skin tests in egg-sensitive individuals (18). Similarly, no lysine-sugar residues have been detected in the feather allergens (9). However, since there is some physicochemical evidence for uncommon interactions between amino groups and sulphhydryl groups in both ovomucoid and soluble feather keratins, the cross-reaction of these allergens with the anti dandruff serum might possibly find an explanation in the existence of pseudo-isothiazoline ring systems in these biologically active proteins, structurally related to the pseudo-isoxazoline hapten shown above. The precipitation reactions with ovomucoid or feather allergens as the antigens could not be inhibited with N-(1-deoxy-1-D-fructosyl)- ϵ -aminocaproic acid.

During some preliminary quantitative assays entire precipitin curves were determined (20) of the antiserum with homologous dandruff allergen, house dust allergen (fraction E) and feather allergens as the antigens. Antibody titers for heterologous house dust antigen, feather extract or normal human serum were considerably lower than for homologous antigen. Inhibition experiments with EACAF were most successful with the homologous antigen-antiserum system, while the control compound ϵ -aminocaproic acid proved far less effective. The amount of EACAF causing 50 per cent inhibition of precipitation of the dandruff allergen at the point of optimal antigen-antibody proportions gave rise to 22 per cent inhibition in a similarly balanced system of house dust allergen-antiserum, and to 10 per cent inhibition of precipitation in the feather allergen-antiserum mixture. The necessity for relatively massive inhibitor concentrations for obtaining detectable inhibition might possibly be due to the fact that only about 10 per cent of EACAF in aqueous solution has been estimated to be in the enolized form (5). In view of the extreme complexity of the antiserum, these quantitative assays were not considered sufficiently accurate to yield fundamentally new information on the antigenic relationship of allergens.

DISCUSSION

The present results unequivocally demonstrate the occurrence of antibody for N-glycosidic lysine-sugar determinants in the anti human dandruff serum and they therefore provide additional evidence for the incorporation of such determinants in purified human dandruff allergen (6, 12). The cross-precipitations of several atopic allergens of unrelated origin with this antiserum need not necessarily be due to the interaction with these particular anti-lysyl-sugar antibodies, however. Bearing in mind that many of the allergens, precipitating with the rabbit anti dandruff serum, were found to share antigenic determinants, and in view of the partial inhibition of precipitin formation by the compound N-(1-deoxy-1-D-fructosyl)- ϵ -

aminocaproic acid—quantitatively (in the equivalence zone) and by gel diffusion—, the results provide a reasonably firm basis for the suggestion that anti-lysyl-sugar antibody is indeed involved in cross-precipitation. With the exception of feather allergens and ovomucoid all the allergens employed have, moreover, been found by chemical analysis to incorporate the relevant N-glycosidic lysine-sugar structures. There is some evidence that the nature of the sugar partner engaged N-glycosidically is inessential for specificity, though the initial sugar configuration may be a factor in determining the ultimate stability of the lysine-sugar residue (5).

Even though the definite identification of the common determinant responsible for the observed cross-reactions may lack absolute proof, the mere demonstration of cross-precipitation of the anti-dandruff serum with several active atopic allergens is of considerable interest in relation to the phenomenon of clinical cross-sensitivity. Again, the results presented here definitely point at some inherent property common to all atopic allergens, causing them to cross-react with the anti dandruff serum, or with an anti house dust allergen serum (15). An objection to the effect that these cross-reactions might be attributed to a common full antigen shared by all the allergen preparations and contained (for instance as a contaminant) in the inoculum used for raising the antiserum cannot easily be levelled at this general conclusion because the chemical composition of cross-reacting allergens from for instance tomato, ipecac and kapok has been proved to be entirely dissimilar from that of the purified human dandruff allergen (4, 14, 16). Chemically, these allergens are only related through similar portions (*i.e.* lysine-sugar structural areas) of otherwise distinct molecules. The basis of this conclusion of shared determinant sites, of course, is that purified allergens have been applied; in contrast, the cross-reactions between crude mould extracts recently reported by *Guibert & Causse-Combes* (19) may just be ascribed to allergenically inactive (polysaccharide) full antigens shared by all the extracts.

It is recognized that the system studied here is too complex for any conclusion on the nature of the structural sites governing the relationship among atopic allergens to be anything but tentative; the anti human dandruff serum has merely been chosen for this study because of the possible etiological role of the dandruff allergen in human atopic disease (4, 6), and because the source of the allergen is sufficiently remote from that of the other allergens applied. The author also realizes that some of the systems studied do not represent adequately balanced antigen-antibody mixtures. However, as a model and a replica of the phenomenon of clinical cross-sensitivity the general results of this investigation may possibly aid in constructing a unitarian theory on the mechanism underlying human atopic disease, while the qualitative relationship among atopic allergens detected here may perhaps be sufficiently alarming to cause a revision of current concepts on the theoretical basis of desensitization therapy.

SUMMARY

Rabbit antiserum for purified human dandruff allergen has been employed for studying its interaction with several different atopic allergens and with synthetic protein-sugar conjugates incorporating N-glycosidically bound sugar. Almost every allergen studied produced precipitation lines during immunodiffusion tests against the anti dandruff serum, while β -lactoglobulin-lactose conjugates and ovalbumin-aldose complexes were also found to precipitate antibody in the anti dandruff serum. These cross-reactions could be partially inhibited with a low-molecular weight ϵ -aminocaproic acid-glucose conjugate. Several cross-precipitating antigens in the allergen preparations were found to share antigenic determinants.

The conclusion is reached that the anti human dandruff serum encompasses anti lysyl-sugar antibody, which may also be responsible for the precipitation of unrelated atopic allergens. The possible significance of these findings is discussed in relation to the phenomenon of clinical cross-sensitivity.

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