CHEMICAL APPROACHES TO PENICILLIN ALLERGY - II

The involvement of a carrier-receptor protein in penicillin allergenesis.

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SUMMARY: Sera from rabbits immunised with penicillin was fractionated on synthetic polymers containing the structural elements of penicillin. Two protein fractions were isolated in homogeneous form and identified as the antibody and the receptor portein. The serum from a sensitive human subject also was shown to contain a receptor protein by employing the same technique. The approximate molecular weight of the receptor protein from the rabbit serum as determined by SDS gel electrophoresis technique was around 60,000.

The exact nature of the penicillin antigen in allergenesis is not known. However, most workers in the field seem to agree that the major antigens leading to allergic reactions are polymeric penicillin impurities in penicillin. It has been demonstrated by Shaltiel *et al*[1] that the antibodies raised against polymeric penicillin and ampicillin[2] bound to serum proteins such as bovine albumin are specific for the polymeric penicillin and not the carrier molecule. Further, they have also suggested that affinity columns containing the purified antibodies raised aganist such antigens could be used to detect the presence of polymeric penicillin impurities in penicillin preparations.

Earlier data from Levine *et al*[3] on the other hand indicate that smaller molecules such as penicillin or the common degradation products of penicillin are determinants in penicillin allergy and give cross reactions in sensitive subjects or animals. In that eventuality it is necessary to postulate the existence of a hypothetical specific penicillin carrier-receptor to supply the bulk of the molecular weight needed to build a molecule of antigeneic dimensions (> 20,000).

The question was reexamined in the present work utilising affinity chromatography of allergic rabbit sera on hydrophobic templates containing the structural elements of penicillin. Three of these templates (TF I, TF II, TF III), "the tail-free" series were synthesised with the carboxyl end of penicillin free and the other three (HF I, HF II, HF III), "the head-free" series were synthesised with the phenyl acetamido end free with 0-7 carbon atoms separating the active moiety from the backbone. The full details of the synthesis of these columns are described elsewhere.[4]

MATERIALS AND METHODS: Five adult rabbits, 3 females and 2 males (average weight 1-2 kgs.), were made allergic to penicillin by injecting 1,00,000 units of penicillin G (Crystapen, Glaxo Laboratories) in one ml saline containing 0.1 ml complete Freund's Adjuvant in multiple places in the foot pads and the back of the animals. The administration of penicillin was repeated bi-weekly for the first four weeks and at weekly intervals there-after.

After 10 weeks the hemagglutination titres of samples of sera of the animals were determined (Table I). The sensitised red blood cells for hemagglutination were prepared according to Josephson[5] with some modifications. A fresh 1.0% suspension of red blood cells in phosphosaline (pH 7.2, 0.05M) was incubated with penicillin G (6 mg/ml of suspension) at 37°C for 30 min, centrifuged and the sensitised RBC was washed twice with 0.8% saline. The washed RBC was resuspended in 0.8% saline to get a 1% suspension of sensitised RBC. One male rabbit from the same stock was used as a control.

TABLE - I

Hemagglutination titres of the rabbit sera

Dilutions	Rabbits
1:20	R_1 (M)
1:1280	R_2 (F)
1:1280	R_3 (F)
1:1280	R_4 (F)
1:40	R_5 (M)

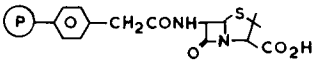
Table representing the hemagglutination titre against the rabbit number, sex of the rabbit in parentheses.

It may be noted that the female rabbits showed good response to sensitisation while the male rabbits were relatively refractory. The sample size is not significant enough to draw conclusion regarding the influence of sex in determining sensitivity.

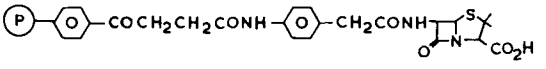
The antisera from the rabbits R_2, R_3 and R_4 showed clear precipitin bands against penicillin immunodiffusion on Ochterlony Plates.

The sera were fractionated using both the types of polymers (TF I, TF II, TF III, HF I, HF II, HF III). All the six polymers completely sequestered both the antibody and the receptor from the sera and the filtrates did not shown any precipitin reaction against penicillin or any hemagglutination response.

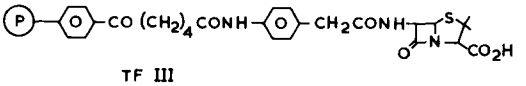
TAIL FREE POLYMERS



TF I

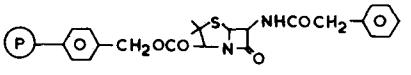


TF II

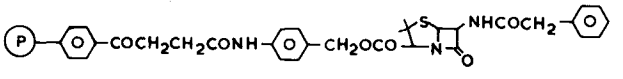


TF III

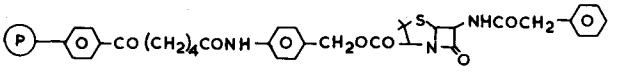
HEAD FREE POLYMERS



HF I



HF II



HF III

However by trial and error the polymer HF III was selected as it was better suited for both absorption and recovery of the protein fractions.

Since the polymer had a tendency to float on water, it was used in batch experiments.

The antiserum (5 ml in 10 ml phospho saline 0.05M, pH 7.2) was stirred with the polymer (2 g) for 1/2 hr at 4°C. The mixture was filtered in the cold and the non-specifically absorbed serum proteins were washed out with three 20 ml portions of 0.05M phospho saline (pH 7.2)(PBS wash). This fraction accounted for virtually all the absorbed serum proteins.

In order to elute the more strongly absorbed proteins a mild denaturing agent such as 0.9M thiourea was used. The polymer was washed with two 10 ml portions of the 0.9M thiourea at 0-4°C with stirring for 10 mts followed by filtration.

Any other proteins not covalently bound to penicillin on the polymer was washed out batchwise with 8M urea.

To cleave the covalently bound hypothetical receptor protein from the polymer along with the penicilloyl moiety the polymer was subjected to hydrogenolysis in 0.05M phospho saline (pH 7.2)(25 ml) at 4°C using platinum (Adam's catalyst 100 mg) with magnetic stirring in hydrogen atmosphere for 1 hr and filtered in the cold.

All the eluates including the filtrate from the hydrogenolysis experiments were dialysed extensively against double distilled water for 48 hrs at 0-4°C with repeated changes of water and freeze dried.

RESULTS AND DISCUSSION: While most of the serum proteins were recovered in the initial 0.05M phospho saline wash very small quantities of protein was isolated from the 0.9M thiourea, 8M urea and hydrogenolysis fractions. The hemagglutination, immunodiffusion and immunoelectrophoretic patterns of these fractions against rabbit antisera are recorded in Table II.

The receptor from the hydrogenolysis fraction was characterised by immunodiffusion in agar against antiserum and also by immunoelectrophoresis. In both the cases a single precipitin band was observed.

The fraction in 0.9M thiourea was demonstrated as the antibody

TABLE II

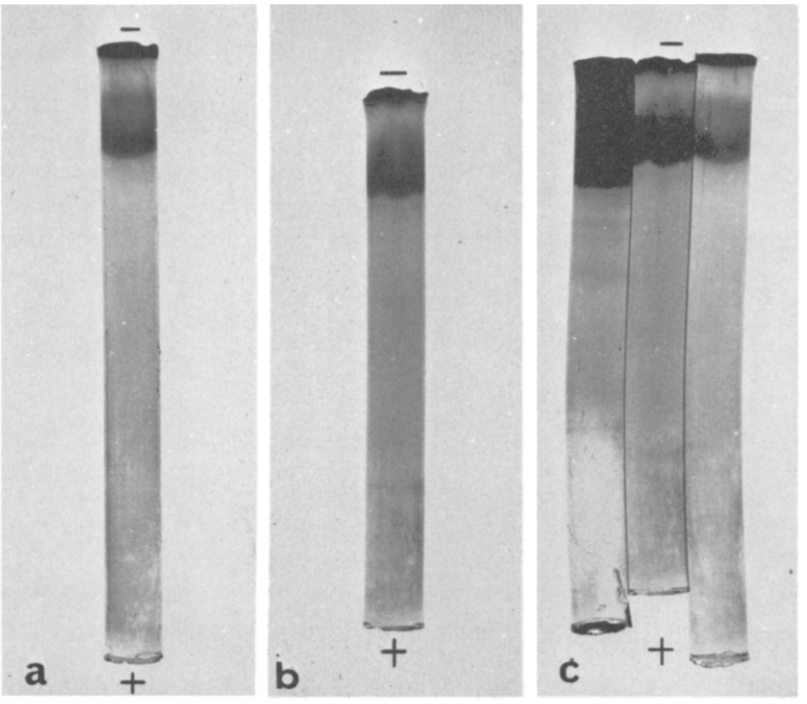
Eluate	Total Quantity of protein µg	Hemagglu-tination	Immuno diffu-sion	Immuno electro-phoresis	Purity - Bands in electro-phoresis
PBS	bulk of protein	-ve	-ve	-ve	-
0.9M thiourea	250	+ve	-ve	-ve	single
8M urea	115	denatured			
Hydrogenolysis	480	-ve	+ve	+ve	single

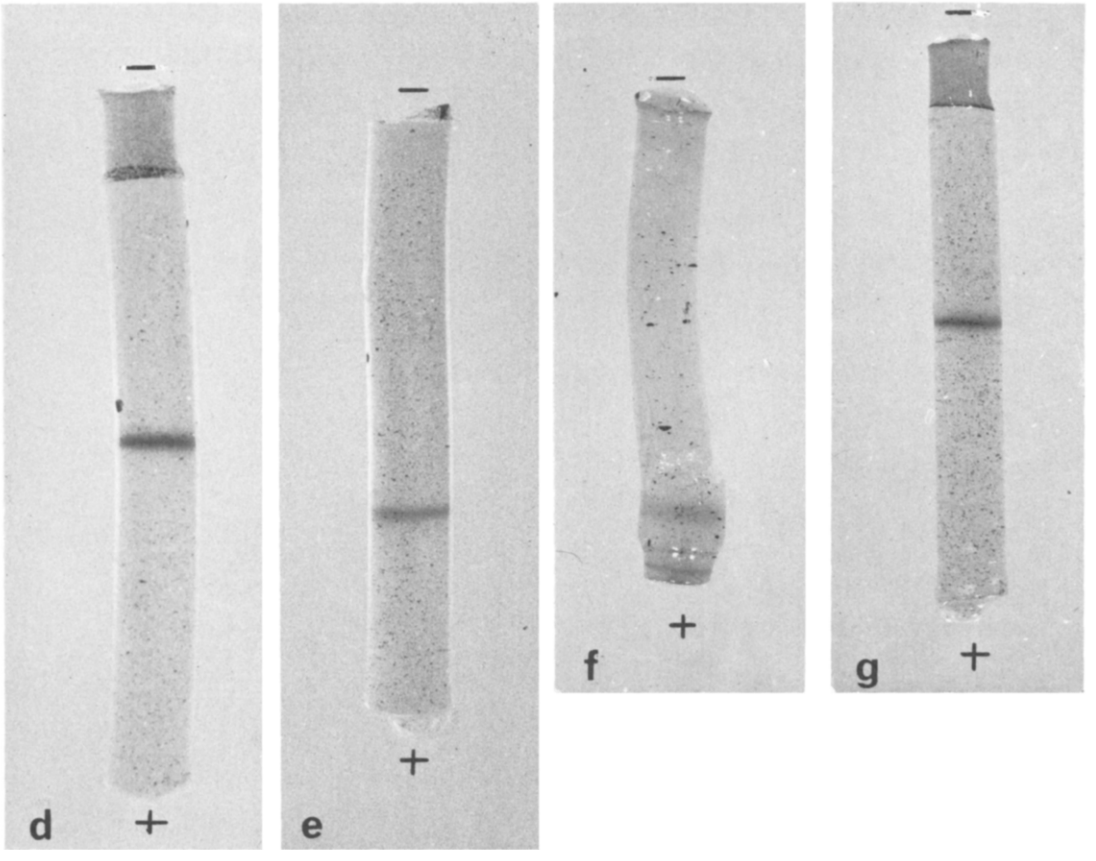
Table representing the various eluates and the tests employed to characterise the proteins.

by a positive hemagglutination titre (1:640 dilution units) and its hapten-specificity by the changed mobility in disc-electrophoresis after incubation with penicillin (Fig. 'd' and 'e' represent the mobility of the antibody before and after incubation with penicillin). Figures 'a' and 'b' represent the mobilities of the receptor proteins from two different rabbits in SDS gel electrophoresis, while 'c' is a comparision of the two receptors and Bovine serum albumin, thereby showing that the receptor proteins have a molecular weight of (~60,000). Figures 'f' and 'g' represent the receptor proteins from rabbit and human sera respectively. The human patient (JB) had a history of penicillin sensitivity for 15 years. The serum from the patient was subjected to identical affinity fractionations.

It is noteworthy that all the three female rabbits had receptor and anti-body of identical mobilities. The control rabbit and non-allergic human subject (CVN) did not give any appreciable protein in the thiourea and hydrogenolysis fractions.

DISCUSSION: Previous work by Shaltiel,[1] Grant,[6] De Weck[7] and others[8-10] appears to have shown the existence of antigenic proteinaceous and polymeric impurities in penicillin preparations. In the light of the present work one has to reexamine the question as to whether these impurities can directly provide the antigen, or do so in combination with a carrier-receptor. Any cross reaction of polymeric benzyl penicillin itself is not


a
b
c


d
e
f
g

a surprising phenomenon, as it still contains the active moiety. But the presence of other protein-bound penicillin antigens cannot be entirely ruled out. The design of present work has been to conclusively establish the presence of a protein in the serum of an allergic subject or animals binding covalently with penicillin and showing in the penicillin-bound form strong specific reactivity, either with the unfractionated antiserum, or with the pure antibody. This in itself can be regarded as sufficient evidence for the involvement of a very specific receptor, at least in certain types of penicillin allergy. The same receptor may perhaps combine with polymeric impurities and convert them into the active antigen. The status of the carrier-receptor in penicillin allergenesis with other determinants as well as polymeric penicillin can only be assessed, if it were possible to isolate it in the penicillin-free form. Some studies are in progress towards attaining this objective with polymers of penicillin analogues.

De Weck et al[7] fractionated penicillin on Sephadex columns and obtained three fractions termed as proteinaceous, penicillin polymer and pure monomeric penicillin fractions. All the three fractions gave cross reactions in sensitive subjects or animals. Any cross reaction of monomeric penicillin in this system would indicate that its role in allergenicity could be that of a hapten only. Nevertheless, some clear cut experiments are needed to settle the argument whether the proteinaceous impurities containing some bound penicillin are primary antigens or merely cross-react with the antibodies.

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