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ENZYMATIC UNMASKING FOR ANTIBODIES OF PENICILLOYL RESIDUES BOUND TO ALBUMIN

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Abstract—After penicillino-therapy, penicilloyl groups, derived from penicillin by rupture of the β lactam ring, are covalently bound to serum albumin to form compounds that have lost all antibiotic activity but possess an allergenic potential. Most of these groups, buried inside the albumin molecule, are non accessible to specific anti-penicilloyl antibodies. After an enzymatic degradation of the albumin, they are unmasked for antibodies recognition which permits their detection. This enzymatic unmasking of penicilloyl groups conjugated to albumin reveals two forms of penicilloyl residues different by their accessibility to antibodies and by their amount and their kinetic of elimination. It raises the problems of the site and mode of the *in vivo* fixation of penicilloyl on albumin and of the pharmacological and toxicological significance of the penicilloyl groups buried inside the albumin molecule.

Allergenic accidents in Man may occur after penicillino-therapy [1] or consumption of products from penicillin-treated animals [2]. Numerous studies [3, 4] underline the major role of penicilloyl-protein conjugates as antigenic determinant. The conjugates, derived from various penicillins by rupture of the β-lactam ring and then covalent binding to proteins, have lost all antibacterial activity but possess immunogenic potential (Fig. 1). Their preparation is quite easy *in vitro*, and used to obtain antigenic derivatives to immunize rabbits for anti-penicilloyl antibodies production. When carrying out this conjugation reaction, then measuring penicilloyl using either chemical methods, isotopic dilution techniques or radioimmunoassay (RIA), authors found several penicilloyl groups bound per molecule of albumin [5–7].

Using a specific radioimmunoassay [7], evidence of *in vivo* formation of minute amounts of such conjugates has been shown previously in Man [8], and animals [9]. However, the concentration of such residues (less than 0.01 penicilloyl group conjugated per molecule of albumin) is very low when compared with *in vitro* fixation.

On the other hand, an abnormal electrophoretic band of "faster" albumin used to appear in human serum of patients receiving high dosage of Penicillin G [8, 10]. In such bisalbuminemia induced by penicillino-therapy, the separation and isolation of the two types of albumin is thus possible. In order to check if the difference of mobility could be due to the fixation of a ligand (i.e. negatively charged penicilloyl group) on ε. NH2 groups, determinations of bound penicilloyl have been performed on both fast and normal albumins isolated from treated patients sera. In this hypothesis a minimum of one penicilloyl group bound per molecule of isolated fast albumin was to be expected. Actually fast albumin

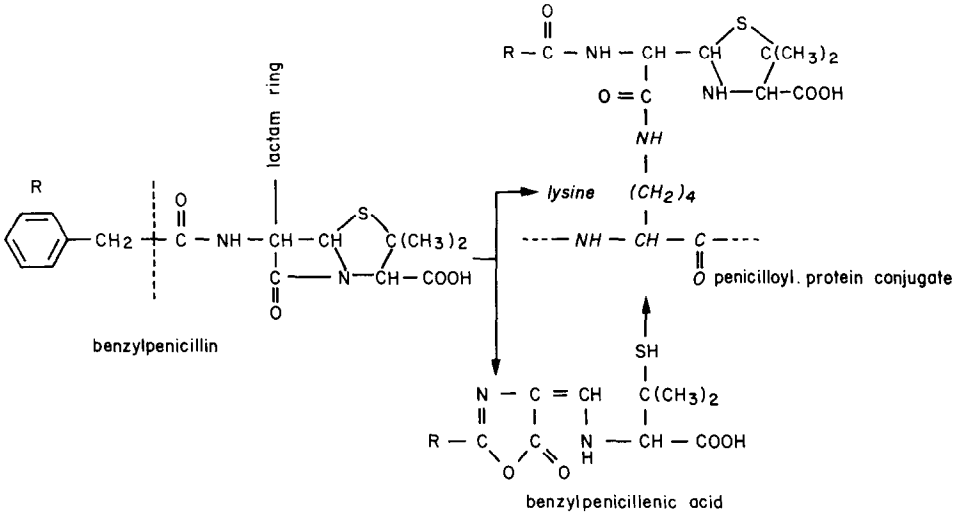

Fig. 1. Formation of the penicilloyl-protein conjugate.

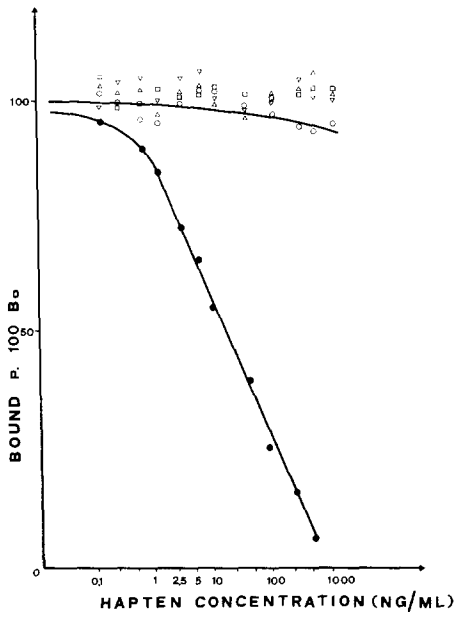

Fig. 2. RIA standard curves obtained with:
○ Benzyl penicillinate of Na (Penicillin G);
△ D Benzyl penicillamine;
□ D L Penicillamine;
▽ Benzyl penicillenic acid;
● Penicilloyl groups in penicilloyl-serum albumin conjugate form.
Similar standard curves are obtained when using an univalent hapten (e.g. penicilloyl ε amino-caproate) for the inhibition of the reaction.

Same standard curves are obtained either in PBS, in albumin solutions (10, 1 or 0.1 mg/ml) or in enzymatically degraded albumin solutions (after heating or neutralization).

molecules contained bound penicilloyl groups in a much greater amount than the normal ones but the number of penicilloyl groups bound per molecule of fast albumin could never be found higher than 0.1, which was too low to explain the faster mobility observed [8].

These observations suggested that this mechanism of *in vivo* fixation of penicilloyl on albumin, could involve more bound penicilloyl groups than those really determined, and that they would be masked inside the albumin molecule. From this hypothesis the idea has been developed that a degradation of the albumin molecule would unmask these penicilloyl groups and make them detectable.

METHODS

Experiment was carried out in pig. After an intramuscular injection of 10 M I.U Penicillin G, blood samples were collected from the carotid artery by a catheter during 8 days. Albumin was precipitated from the serum with a solution of 1% trichloroacetic acid (TCA) in ethanol [11], then purified on a G 50 Sephadex Column.

Penicilloyl groups fixed to albumin were measured with the radioimmunoassay previously described [7]. The antisera were obtained from rabbits by immunization with penicilloyl-bovine γ-globulin (BGG). The sera were tested for antibody titer and the strongest antiserum was cleared of anti-BGG antibodies with glutaraldehyde cross-linked bovine serum albumin (BSA)-BGG immunoadsorbent. The tracer was obtained by coupling the penicilloyl groups to BSA previously labelled with ^{125}I. The specific activity of the ^{125}I-BSA-penicilloyl conjugate was about 70 mCi/mg of benzyl-penicilloic acid. Its immunoreactivity ranged between 90 and 95 per cent of the total radioactivity. The competition reaction between labelled antigen, unlabelled hapten and antiserum was carried out overnight at room temperature in phosphate buffer (pH 7.4) containing 0.5% human serum albumin. After 4 hr incubation at 4° with anti-rabbit sheep serum, the labelled antibody–(penicilloyl-iodinated BSA) complex was completely precipitated. After centrifugation, the supernatant was discarded and the radioactivity of the pellet was measured with a γ-scintillation spectrometer. However, in order to have standard penicilloyl under the same form as those of the experimental samples, a standard penicilloyl-serum albumin conjugate was prepared and used as unlabelled antigen instead of the penicilloyl ε aminocaproate as in the original RIA. Standard curve obtained with this conjugate (Fig. 2) shows the binding inhibition measured vs logarithm of unlabelled penicilloyl added. In fact, identic results are obtained whether the inhibition is realized with penicilloyl groups conjugated to albumin or with an univalent hapten (e.g. penicilloyl ε amino caproate) which confirms our first observations [7]; moreover it can be seen that the anti-serum is specifically anti-penicilloyl and that no cross reaction occurs with penicillin G and other derivatives. The non specific binding obtained with normal rabbit serum is lower than 2 per cent of the total activity introduced. The detection limit is sufficient for the intended applications, and we purposely prepared a tracer of moderate specific activity to increase its reliability and shelf-life. The different biological materials have no effect on the assay. Determination of penicilloyl groups can thus be made directly in these biological samples without previous extraction, after suitable dilution.

Albumin fractions and their dialysates (after 48 hr dialyse against distilled water) were tested for the presence of Penicillin. The method used was the classical diffusion on agar plates with *Bacillus stearothermophilus* as test organism.

Microbiological and radioimmunological assays were both performed in solutions containing 10, 1 and 0.1 mg of albumin per ml. Each determination was made using a standard curve established at the same albumin concentration. For RIA, it was observed that within this range, the albumin concentration did not interfere with the determination. In the conditions used the lowest quantities measurable were about 3 ng of Penicillin with the microbiological assay and 0.5 ng of penicilloyl with the RIA.

Degradations of isolated albumin fractions were realized either by Pronase (B grade-Calbiochem) or subtilisin Carlsberg (Sigma), both bacterial proteases, or by rabbit Cathepsin D prepared according to Lapresle and Webb [12]. Incubation with pronase

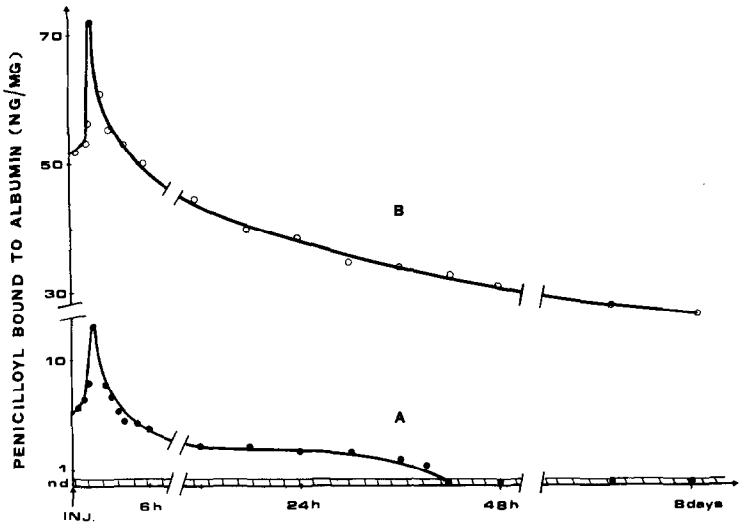

Fig. 3. Concentration of penicilloyl groups covalently bound to pig serum albumin after one I.M. injection of 10 M I.U. Penicillin G (ng of penicilloic acid per mg of albumin):
A—before degradation of albumin;
B—after unmasking by complete enzymatic degradation of albumin molecule using either pronase or subtilisin Carlsberg.
n.d.—non detectable. The limit of detection of the RIA used is 0.5 ng of penicilloyl group per mg of albumin.

or subtilisin was allowed to set 5 hr at 37° in 0.01 M phosphate buffer pH 7.4–0.15 M NaCl (PBS), containing 1 mg of albumin and one protease unit of enzyme per ml. The reaction was stopped by immersing the tubes in boiling water for two minutes.

Incubation with cathepsin D was allowed to set 6 hr at 37° and pH 3.5 in 0.15 M NaCl containing 10 mg of albumin and five proteolytic units per ml. The reaction was stopped by neutralizing the solution.

RIA of penicilloyl groups is carried out before and immediately after degradation of albumin samples, in the same tubes or after convenient dilutions in PBS. Besides, aliquots of each sample were precipitated by 2% TCA before and after hydrolysis. The degradation rate was estimated from the ratios of the two pellet protein contents measured with the biuret reagent [13].

RESULTS

Concerning albumin degradation, it appears that, in conditions used, no material can be precipitated by 2% TCA after proteolysis either by pronase or subtilisin. After Cathepsin D hydrolysis, the ratio material precipitated by 2% TCA vs total amount of albumin present before degradation is approximately 20 per cent. It can be considered that, with this criterion, degradation by pronase or subtilisin is "complete" or at least more complete than degradation by cathepsin D.

Penicilloyl determinations show (Fig. 3A, B) that after complete degradation of albumin either by pronase or subtilisin, penicilloyl concentration measured is about 10 times greater than in non degraded samples.

A limited degradation of albumin, such as splitting by rabbit cathepsin D, liberates penicilloyl groups, but at a twice lower rate. The unmasking of penicilloyl groups for RIA determination is thus related to albumin degradation. On the other hand it appears that the "unmasking" procedure reveals not only an increase of the amount of penicilloyl groups detected all along the depletion time, but also a much longer persistance of these residues after penicillino-therapy. Two days after injection no penicilloyl could be detected at levels as low as 0.5 ng/mg albumin while after enzymatic degradation of albumin, penicilloyl residues are still present 8 days later at levels higher than 20 ng/mg albumin. It thus appears that this unmasking of penicilloyl groups for RIA determination does not only increase the sensitivity of the method but it permits to detect two different types of residues, the ones directly accessible to antibodies and rapidly eliminated, the others persisting in much greater quantities and revealed only after albumin degradation.

Interferences on RIA determinations can occur from a proteolysis of antibodies by the remaining pronase or subtilisin, as RIA incubation and proteolysis are both performed at pH 7.4 whereas Cathepsin D has no enzymatic activity at this pH. Slight false positive results are, indeed, observed on control blank samples of albumin isolated from untreated animals sera and degraded by pronase or subtilisin. Heating samples 2 mn at 100° before the RIA destroy remaining proteases.

Using this mode of operation, the RIA specificity for penicilloyl determination in degraded albumins has been confirmed by several control tests in different conditions. Determinations were performed on control blank samples of albumin from untreated animals and on the same samples after enzymatic degradations. In albumin solutions as well as in the

heated hydrolysates after pronase or subtilisin degradation or in the neutralized hydrolysate after cathepsin degradation, negative results were obtained. That is to say that no inhibition of the antigen antibody binding reaction occurs and no false positive appear in the RIA determination due to the degradation process. On the other hand, samples of control blank albumin or of its different hydrolysates have been fortified with determined quantities of standard pencilloyl (from 0.5 to 50 ng of penicilloyl per mg of albumin). Penicilloyl has been added either as penicilloyl-albumin conjugate or as penicilloyl ε amino-caproate. In every case the total penicilloyl added has been recovered with the RIA determination. Enzymatic degradation of albumin brings no interference, and no cross reaction can then be observed in albumin hydrolysates samples even if the inhibition of the reaction is realized with a univalent hapten as penicilloyl ε amino-caproate which can thus be used in this case too as unlabelled antigen for the specific determination of penicilloyl groups.

On the other hand, neither before nor after degradation did microbiological assays show any antibiotic activity in albumin samples. Moreover after dialyse, no antibiotic activity was found in the dialysate.

DISCUSSION

This RIA using high-affinity and specific antipenicilloyl antibodies has permitted to demonstrate the presence of trace amounts of penicilloyl-albumin conjugates in serum after penicillino-therapy. The absence of antibiotic activity in experimental albumin fractions and in their dialysates shows that they contain neither free, nor uncovalently bound penicillin. This kind of binding studied concerns only covalently bound penicilloyl groups and thus differs from the classically described reversible fixation of penicillin to albumin [14, 15] where, after treatment, larger amounts of penicillin are bound and then gradually released in keeping the whole antibiotic activity.

The "masking phenomenon" observed for *in vivo* fixation of penicilloyl into albumin can of course be due to the method of detection that involves reaction of penicilloyl groups with antibodies. Steric limitations could permit only few antibody molecules to bind with penicilloyl regardless of how many penicilloyl groups the albumin conjugate would contain. After degradation into fragments, more opportunities for binding to antibody exist. At this stage, it must be underlined that our immuno-detection of bound penicilloyl is a well adapted tool. The biological mechanism, e.g. the recognition of a penicilloyl antigenic site by a specific antibody, used for the determination, is the same as occurs *in vivo* in an allergenic reaction of the organism to penicilloyl conjugates. Therefore, either accessibility or non-accessibility of penicilloyl antigenic sites, located in the interior of the albumin molecule, can be supposed to be the same whether the antibodies are rabbit anti-penicilloyl prepared for the assay, or human secreted by the organism after a previous antigenic agression. The masking of penicilloyl groups for antibodies, observed with rabbit antibodies in the determination process, would occur in the same way with human IgG or especially IgE antibodies in the recognition of penicilloyl deeply bound to foreign proteins entering the organism. The masking observed has thus a real pharmacological or clinical significance and is not a failure of the method; moreover it raises the problem of the allergenicity of penicilloyl groups buried inside protein molecules.

On the other hand, the present work indicates that, in such formation of penicilloyl-albumin conjugates, a previous complete degradation of albumin is necessary to determine all the penicilloyl groups covalently bound. The fact that most of the penicilloyl groups are, *in vivo*, deeply fixed inside the albumin molecule suggested the hypothesis that fixation might happen on reactive lysine residues of the native albumin molecule. The masking of the penicilloyl thus fixed would appear later on either when the molecule folds to get its tertiary structure or would result from protein–protein interactions.

Confirmation of this observation and of this hypothesis has been brought by study on bisalbuminemia [8] and by the work of Knight and Green [16] published since then. Enzymatic degradation of fast albumin molecules has increased the amount of bound penicilloyl groups detected to one penicilloyl group per molecule so that it became confirmatory. On the other hand Knight and Green, studying the interaction of dinitrophenyl groups bound to Bovine Serum Albumin with univalent fragments of anti-dinitro phenyl antibody, have described the same phenomenon and checked a similar hypothesis. They demonstrated that Dnp groups bound to albumin were largely unaccessible to univalent anti-Dnp antibodies. Their availability was much increased after proteolytic cleavage by digestion with pepsin. Moreover they have shown that the availability of Dnp groups bound to albumin was markedly increased when the albumin molecule was unfolded at acidic PH values. They suggested that the hindrance to the binding of antibody by Dnp-albumin would arise from protein–protein interactions after fixation on reactive lysine residues located in clefts between the globular sub-domains of the albumin. Moreover these authors have observed the same limited degree of binding with Dnp as we measured with penicilloyl (1 group per molecule of albumin) even at high concentration of reactants. This limitation observed in both cases after proteolytic cleavage suggests that the same mechanism is involved.

As the amount of penicillin involved in the formation of penicilloyl-albumin conjugate is extremely small, it affects a very low proportion of the administered drug and should have no appreciable effect on the bio-availability of the antibiotic.

On the opposite such a formation of penicilloyl-protein conjugates, even in trace amounts, after Penicillino-therapy, may have implications for human health. These metabolites whose allergenic action is well known, may be responsible for the anaphylactic accidents observed. In the case of veterinary medicine, they constitute the true active residue present in the tissues of penicillin treated animals, that is potentially dangerous for human consumers. On the other hand the masking for the antibodies of most of the penicilloyl groups of the

conjugate, into the albumin molecule, modifies the pharmacokinetic profile of these metabolites. Two forms of penicilloyl residues are thus observed: one form directly accessible to antibodies and rapidly eliminated, the other revealed only after albumin degradation, that remains in much greater quantities for several weeks. The existence of such a compartment increases the implications of veterinary penicillino-therapy. The risk of ingestion of penicilloyl residues in animal products exists if a sufficient withdrawal time of several weeks is not respected between treatment and slaughtering.

Concerning the pharmacological and toxicological implications of these residues, the masking phenomenon raises the problem of the significance of buried and hidden penicilloyl groups. The classical allergenic action of penicilloyl-protein conjugates as it is described [3, 4] may be, in fact, different if the penicilloyl groups are fixed on the outside of the molecule and therefore accessible to IgG and IgE antibodies, or masked inside. Nevertheless, in the case where anaphylactic accidents would not result from bound penicilloyl hidden into albumin molecules, the allergenic risk persists as long as these penicilloyl groups are gradually unmasked for antibodies and by *in vivo* release, i.e. during the albumin turn-over.

This masking phenomenon may be of a general interest in biochemical pharmacology or in clinical chemistry. The metabolic study of a drug and the determination of its pharmacokinetic profile require specific and sensitive analytical methods for the parent compound but also for its main active metabolites. In the case of penicillin, the allergenic penicilloyl groups cannot be detected by the classical microbiological assay of Penicillin; that is why a specific RIA was prepared for their detection. RIA because of its specificity and sensitivity is one of the best analytical methods for studying ligands bound on proteins; in the case of penicilloyl groups, it is the only one available. However, this example shows that this method needs to be used with much precautions as ligands can be located in places not accessible to antibodies, and are thus not detected unless a previous degradation of the protein carrier is made.

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