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DRUG–PROTEIN CONJUGATES—IX

IMMUNOGENICITY OF CAPTOPRIL–PROTEIN CONJUGATES

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Abstract—The immunogenicity of captopril (CP), conjugated to heterologous proteins, was investigated in male New Zealand White rabbits by monthly injections of CP–protein conjugates in Freund's Complete Adjuvant. Anti-CP antibody activity was readily detected by immunodiffusion in sera of rabbits immunized with the amide-linked CP-HSA (23:1) conjugate. Hapten inhibition studies revealed that the antigenic determinant contained CP and a lysine residue from the protein carrier. When rabbits were immunized with disulphide-linked CP–*S*–*S*–HSA (9:1) and CP–*S*–*S*–KLH (160:1) conjugates, anti-CP antibody activity was detected by a sensitive ELISA method, but not by immunodiffusion and radioligand binding assays. The specificity of the serum IgG anti-CP activity after immunization with disulphide-linked CP–*S*–*S*–protein conjugates was confirmed since anti-CP activity was inhibited by preincubation of the antisera with CP conjugated to an unrelated protein carrier (CP–*S*–*S*–OVA), but not by the corresponding unconjugated protein, nor by penicillamine–*S*–*S*–protein conjugates. These results show that disulphide-linked CP-protein conjugates are sufficiently stable to induce humoral (B lymphocyte) anti-hapten responses under the experimental conditions employed. In a separate study, delayed-type skin hypersensitivity reactions to topically applied CP were demonstrated in the guinea pig. The specific and sensitive immunochemical technique (ELISA) described here could be useful in future studies for determining whether or not patients taking CP produce antibodies to the drug.

Captopril (D-3-mercapto-2-methyl-propanoyl-L-proline; CP)† is an orally active angiotensin-converting enzyme inhibitor used in the treatment of hypertension and congestive heart failure [1–4]. Chronic administration of CP can lead to skin rash, fever, dysgeusia, proteinuria, ulcers, leucopenia, agranulocytosis and nephrotic syndrome in some patients [1, 2, 4]. Adverse reactions are observed most frequently in those patients receiving high doses and with underlying renal diseases and/or collagen–vascular diseases [5, 6]. The nature and time course of CP adverse reactions are consistent with there being an underlying immunological mechanism [7]. Moreover, immune complexes have been detected in the glomerular basement membrane in renal biopsy samples from patients given CP [7, 8]. It has therefore been suggested that CP or one of its metabolites, may function as a hapten in inducing hypersensitivity reactions.

The current understanding of drug-induced hypersensitivity is based on the "hapten hypothesis", central to which is the assumption that drugs, and low mol. wt substances in general, must become covalently bound to endogenous macromolecules to be recognised by the immune system [9]. We have previously studied the metabolism of CP and found that the drug becomes extensively and covalently bound to plasma proteins both *in vitro* and *in vivo* [10]. Binding of CP is mainly to albumin, via disulphide linkage [11], which is a relatively labile covalent bond. Thus, after intravenous administration into rats, CP–*S*–*S*–plasma protein conjugates were cleared rapidly from plasma and CP appeared in urine mainly as CP–cysteine mixed disulphide [10]. Further studies showed that dissociation of CP–*S*–*S*–plasma protein conjugates can be induced by a spontaneous thiol–disulphide exchange reaction with endogenous thiols such as cysteine and glutathione [12]. Therefore, the ability of CP to act as a hapten may be influenced by the relative rates at which the CP-protein conjugates form and dissociate *in vivo*.

To determine whether the disulphide bond of CP–*S*–*S*–protein conjugates is sufficiently stable to elicit an immune response to CP, we have investigated the *in vivo* immunogenicity of a number of CP–protein conjugates (disulphide- and amide-linked) in experimental animals. A range of test systems have been employed to investigate both humoral and cell-mediated immunity in this study.

MATERIALS AND METHODS

[^{14}C]Captopril (4.66 μCi/mg) labelled at the amide-carbonyl carbon, captopril and other authentic standards of captopril disulphide, captopril–

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† Abbreviations: CP, captopril; CP–*S*–*S*–HSA, disulphide-linked captopril conjugate with human serum albumin; CP–*S*–*S*–KLH, disulphide-linked captopril conjugate with keyhole limpet haemocyanin; CP–*S*–*S*–OVA, disulphide-linked captopril conjugate with ovalbumin; IgG, immunoglobulin G; BSA, bovine serum albumin; HSA, human serum albumin; KLH, keyhole limpet haemocyanin; OVA, ovalbumin; SDS, sodium dodecyl sulphate; ELISA, enzyme-linked immunosorbent assay.

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cysteine mixed disulphide and captopril–glutathione mixed disulphide were supplied by the Squibb Institute, NJ, U.S.A. *S*-[prolyl-3,4-^{3}H]-acetyl captopril (0.21 Ci/mg) was from New England Nuclear, Boston, MA. Bovine serum albumin (fraction V, BSA), human serum albumin (fraction V; HSA), chicken ovalbumin (grade V, OVA), keyhole limpet haemocyanin (KLH), agarose (Type II), DL-dithiothreitol (DTT), D-penicillamine, penicillin G (benzylpenicillin) and *o*-phenylenediamine (OPD) were obtained from Sigma Chemical Co., London, U.K. Micro-ELISA plates (Falcon 3912-MicroTest III) were obtained from Becton Dickinson Labware, Oxford, U.K. Goat anti-rabbit IgG and horseradish peroxidase-labelled rabbit anti-goat IgG were obtained from Miles Laboratories, Slough, U.K. Dialysis tubing was obtained from Medicell Ltd., London, U.K. Silica gel thin-layer chromatography plates (Merck no. 5735, 20 × 20 × 0.02 cm) and other general reagents and chemicals were obtained from British Drug Houses, Poole, U.K. Scintillation fluid (NE 260) was obtained from Nuclear Enterprises, Edinburgh, U.K. All solvents were redistilled before use. Freund's Complete Adjuvant was obtained from Sigma Chemical Company, U.K.

Synthesis of disulphide-linked captopril–protein conjugates. Captopril–protein conjugate, linked through a disulphide bond (Fig. 1), was synthesized by the following method: 150 mg of protein (HSA, BSA, OVA or KLH), 215 mg of CP and [^{14}C]CP (1 mg, 4.66 μCi/mg) were dissolved in 10 ml of 0.1 M dipotassium hydrogen phosphate containing 7 M urea solution. The mixture was incubated at 37° for 2 hr. Iodine (0.5 M, in 95% ethanol) was added dropwise to the stirred mixture and the pH was maintained between 6 and 7. The mixture was then allowed to stand at room temperature for 1 hr. Excess iodine was removed by the addition of sodium thiosulphate. The mixture was dialysed overnight against 5 l. of sodium phosphate buffer (0.1 M, pH 7.4; containing 0.1% sodium dodecyl sulphate, SDS), followed by further dialysis with sodium phosphate buffer (0.1 M, pH 7.4) only. The resulting CP–protein conjugate was freeze-dried and its epitope density determined as described below. Disulphide-linked conjugates of D-penicillamine with proteins (OVA, HSA, BSA) were prepared by the same method.

Synthesis of amide-linked captopril–protein conjugates. Amide-linked CP–HSA and CP–BSA conjugates formed from [^{14}C]CP–disulphide (Fig. 1) were synthesized as follows: [^{14}C]CP–disulphide (116 mg, 0.02 μCi/mg) was dissolved in 4.2 ml of dry 1,4-dioxan and tri-*n*-butylamine (0.192 ml) added. The solution was cooled to 11° and isobutylchloroformate (50 μl) was added. The mixture was maintained at 10° for 30 min, with occasional mixing. At the same time HSA or BSA (326 mg) was dissolved in 8.6 ml of distilled water, to which was added 1 M sodium hydroxide (0.33 ml) and 1,4-dioxan (5.8 ml). The [^{14}C]CP–disulphide solution was then added slowly to the protein solution. The final reaction mixture was shielded from light and left at room temperature for 4 hr. The mixture was dialysed as described previously for the disulphide-linked conjugates, and then freeze dried.

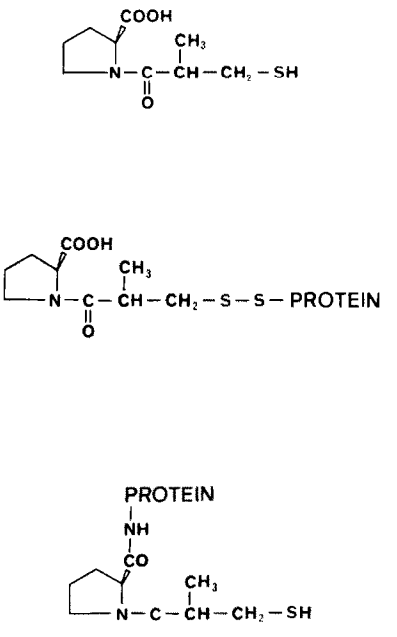

Fig. 1. Structures of captopril, disulphide-linked captopril–protein and amide-linked captopril–protein conjugates.

Finally, the disulphide bond of the protein conjugated CP–disulphide was reduced by dissolving the synthesized conjugate (100 mg) in 5 ml of sodium phosphate buffer (0.01 M, pH 7.0) containing 5 mM dithiothreitol (DDT). At this concentration, DTT has been shown to dissociate the disulphide bond of captopril [13].

$$\text{Protein–CP–}S\text{–}S\text{–CP} + \text{DTT} \rightarrow \text{Protein–CP–SH} + \text{CP–SH}$$

The solution was left at room temperature overnight and dialysed as previously described. Dialysis resulted in removal of 49.24 ± 5.27% of [^{14}C] material from the conjugate mixture.

By this method, *N*-acetyl-lysyl [^{14}C]CP–disulphide was also synthesized, using *N*-acetyl lysine in place of the protein and characterised by mass spectrometry (M^+ 601) [14].

Determination of drug–protein ratios of [^{14}C]*captopril–protein conjugates.* The drug–protein ratios (epitope densities) of the [^{14}C]CP–protein conjugates (disulphide-linked and amide-linked) were determined by equilibrium dialysis in the presence of sodium dodecyl sulphate (SDS), as described previously [10]. The amount of [^{14}C]CP covalently bound was calculated from the amount of non-dialysable radioactivity that remained in the dialysis bag, and the protein content determined [15].

Immunization of rabbits with drug–protein conjugates. Male New Zealand White rabbits (1.5–2 kg) were immunized by monthly injections of drug–protein conjugates (3 mg) emulsified in Freund's Complete Adjuvant into intramuscular and subcutaneous sites [16]. Blood was obtained prior to immunization and at 10 and 14 days after each injection, and clotted overnight. Serum was then obtained by centrifugation (2000 *g*) at 4° for 10 min.

Immunodiffusion assay for antibody activity. Immunodiffusion was by the method of Ouchterlony [17] with minor modifications, as described previously [14].

Radioligand binding assay for antibody activity. A stock solution of *S*-[prolyl-3,4-^{3}H]-acetyl-[^{3}H]CP (0.2 μCi/ml) was prepared in a potassium phosphate buffer–saline solution (PBS; 0.01 M, pH 7.0) containing 0.1% of bovine serum albumin (BSA). Aliquots (50 μl) of this stock solution were pipetted into glass radioimmunoassay tubes. Several dilutions (1:50, 1:100, 1:500 and 1:1000) of the serum raised against the CP–*S*–*S*–HSA conjugate or amide-linked CP–HSA conjugate were prepared with the PBS solution with 0.1% BSA. The diluted serum (100 μl) was added, followed by either 50 μl of PBS solution (with 0.1% BSA) or 50 μl of captopril disulphide (1 ng in 50 μl) prepared in the PBS solution with 0.1% BSA. The solutions were incubated at 4° overnight.

After the incubation period, 100 μl of a 0.5% BSA solution (in PBS) was added, followed by 1 ml of a dextran-coated charcoal solution (in PBS solution, 0.01 M, pH 7.0). The mixture was left at 4° for 10 min, then centrifuged (2000 *g*) at 4° for 10 min. The supernatant was then mixed with 4 ml of scintillation fluid and the amount of radioactivity present was determined by liquid scintillation spectrometry in a Packard-Tri-Carb 4640 scintillation counter. Counting efficiency was determined by automatic standardisation with previously prepared quench curves.

In other radiobinding studies, [^{3}H]CP was prepared by incubating the acetyl-[^{3}H]CP with 0.1 M sodium hydroxide. This was confirmed by thin-layer chromatography with authentic standards of captopril [10]. After neutralisation with 0.1 M hydrochloric acid, the [^{3}H]CP released was used immediately for the radiobinding studies. Similarly, [^{3}H]CP disulphide was obtained by leaving the released [^{3}H]CP to stand at room temperature, after oxygen had been bubbled through the solution. The formation of [^{3}H]CP disulphide was again confirmed by thin-layer chromatography. The [^{3}H]CP disulphide was also used in the radioligand binding studies.

Enzyme-linked immunosorbent assay (ELISA) for antibody activity. Captopril-specific antibody (IgG) in the sera of rabbits immunized with the disulphide-linked captopril–protein conjugates (CP–*S*–*S*–HSA/CP–*S*–*S*–KLH) was determined by ELISA. Microtiter plates were coated overnight at 4° with 1 mg/ml of CP–*S*–*S*–OVA conjugate (epitope density 7:1) or OVA in 0.05 M phosphate buffer, pH 7.2 (125 μl/well). The plates were then washed three times with 0.15 M phosphate-buffered saline (PBS; pH 7.2) containing 0.05% Tween 20 (PBS–Tween) and shaken dry. All subsequent washes were performed in the same way, and all subsequent incubations were for 1 hr at 37° in a moist chamber. All dilutions of sera and antisera were in PBS–Tween. Each well was then successively incubated with the following, with washing between each step: 100 μl of test serum, serially diluted down columns in duplicate; 100 μl of goat anti-rabbit IgG (diluted 5000:1); 100 μl of peroxidase-labelled rabbit anti-goat IgG (diluted 5000:1); 100 μl of substrate solution containing 0.1% hydrogen peroxide (30% w/v) and 400 μg/ml of *o*-phenylenediamine dihydrochloride (OPD) in 0.15 M citrate–phosphate buffer (pH 5.0). The enzyme–substrate reaction was terminated after 5 min by addition of 50 μl of 25% sulphuric acid to each well. Absorbances were read at 490 nm by a dual wavelength automated plate reader (Dynatech MR600), with the reference wavelength set at 630 nm. Antibody titres were calculated as dilution of antiserum giving half-maximal end point absorbance. Anti-CP activity was defined as antibody titre following coating of plates with CP–*S*–*S*–OVA, when no activity was detected against OVA alone. The intra-assay coefficient of variation between 96 wells on the same plate for IgG anti-CP activity (antiserum dilution 1:5000) was 2.6%). The inter-assay variation, calculated as the average coefficient of variation between duplicate treatments on each of three separate plates, was 4.3%.

Contact sensitivity test for cell-mediated immunity to captopril in the guinea pig. Contact sensitivity reactions to captopril were studied by the guinea pig maximization test [18]. Briefly, male guinea pigs (300 g) were given a subcutaneous injection of the drug (in Freund's Complete Adjuvant) at the back of the neck, followed by a topical application of the drug a week later. The guinea pigs were then challenged with another topical application of the drug at a different site on the flanks 2 weeks later. The challenged site was studied 24 hr after the patch was removed. The criteria for an allergic response were constituted by redness and swelling of the skin. The allergic reactions were scored on a three-point scale: 0, no reaction; 1, mild redness, and 2, intense redness and sometimes swelling of the skin. In order to avoid bias in judgement, the assessment procedure was designed in such a way that the assessors examined the animals at random, withour prior knowledge of the type of treatment each animal had received. The procedure was also performed with penicillin G in place of CP as a positive control.

RESULTS

Immunodiffusion

Following immunization of rabbits with the amide-linked CP–HSA (23:1) conjugate, serum antibodies against amide-linked CP–HSA (23:1) and CP–BSA (36:1) conjugates, but not against unconjugated HSA and BSA, were detected by formation of precipitation lines in immunodiffusion assays, indicating the presence of anti-CP antibodies in the antisera (results from three rabbits). The absence of anti-HSA activity was presumably due to the high hapten density (23:1) of the amide-linked CP–HSA used for immunization, which may have masked native carrier determinants. The immunoprecipitation reaction between the antiserum and the amide-linked CP–HSA was inhibited by pre-incubation of the antiserum with *N*-acetyl-lysyl-CP disulphide (1 mg/ml) but not with CP, CP–disulphide, or HSA, thus demonstrating that the antigenic determinant incorporates both CP and a lysine residue of the protein.

Following immunization of rabbits with the disulphide-linked CP–*S*–*S*–HSA (9:1) (four rabbits),

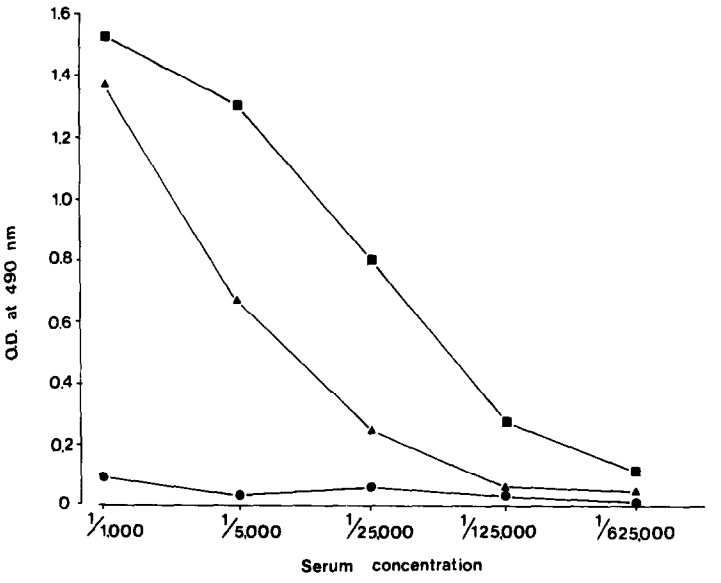

Fig. 2. Time course of the appearance of CP-specific antibody (IgG) in the serum taken on day 14 after the first (▲) and day 14 after the second (■) injection (day 44) of disulphide-linked captopril–KLH conjugate (CP–*S*–*S*–KLH), compared to the preimmunization serum (●) of the rabbit. The CP-specific antibody activity was measured by ELISA.

anti-CP antibodies could not be detected by immunodiffusion. Precipitation lines were detected between the antisera and both CP–*S*–*S*–HSA and HSA, indicating an anti-protein carrier activity. The precipitation lines so-formed were confluent, indicating identity of antigenic determinants. Formation of precipitation lines with CP–*S*–*S*–HSA was not inhibited by pre-incubation of antisera with CP and CP–disulphide. Since we were unable to detect anti-CP disulphide-linked CP–protein conjugates by immunodiffusion, we then employed the more sensitive radioligand binding assay and the very sensitive ELISA to screen for anti-CP activity in these sera.

Radioligand binding

Sera from rabbits immunized with CP–*S*–*S*–HSA and CP–*S*–*S*–KLH showed no specific binding to acetyl-[^{3}H]CP, [^{3}H]CP and [^{3}H]CP-disulphide. Some non-specific binding (approx. 5%) of [^{3}H]CP to the antisera was detected, which was probably due to covalent linkage of the radioligand to proteins in the assay medium.

ELISA

By coating microtitre plates with CP–*S*–*S*–OVA (7:1) we were able to demonstrate IgG anti-CP antibody in the sera of four rabbits immunized with CP–*S*–*S*–HSA (9:1) and three rabbits immunized with CP–*S*–*S*–KLH (160:1). No significant anti-OVA activity was detected when plates were coated with unconjugated OVA.

Figure 2 shows the time course of the appearance of CP-specific IgG in the sera of rabbits immunized with CP–*S*–*S*–KLH. It is evident that circulating IgG anti-CP was detected after injection of the CP–*S*–*S*–KLH conjugate. The concentration of IgG anti-CP increased after a second injection (booster) of CP–*S*–*S*–KLH, but no further increases in anti-CP activity were detected following subsequent boosts. Similar results were observed with rabbit antisera raised against CP–*S*–*S*–HSA.

The anti-CP IgG titres in rabbits immunized with CP–*S*–*S*–KLH were generally higher than rabbits immunized with CP–*S*–*S*–HSA. As shown in Figs. 3a and b, the anti-CP titres varied considerably between individual animals, after immunization with CP–*S*–*S*–HSA And CP–*S*–*S*–KLH, respectively. The specificity of the IgG antibody activity for CP was confirmed by inhibition studies. The binding of IgG antibodies in antiserum raised against CP–*S*–*S*–HSA, to CP–*S*–*S*–OVA, was inhibited in a dose-dependent manner following preincubation of the antiserum (1:5000 dilution) with CP–*S*–*S*–OVA (7:1) and CP–*S*–*S*–KLH (160:1). Preincubation with the carrier proteins (OVA, KLH) or disulphide-linked penicillamine-protein conjugates with OVA (5:1), did not inhibit the specific binding (Fig. 4). Similarly, the end-point activity of the CP–*S*–*S*–KLH antiserum was decreased by preincubation with CP–*S*–*S*–HSA and CP–*S*–*S*–OVA but not with the carrier proteins (HSA, OVA) or penicillamine–protein conjugates, PA–*S*–*S*–HSA, (14:1) and PA–*S*–*S*–OVA (5:1) (Fig. 5).

The binding of IgG antibodies in antiserum raised against CP–*S*–*S*–HSA or CP–*S*–*S*–KLH to CP–*S*–*S*–OVA, was inhibited by preincubation of the antisera (1:5000) dilution for CP–*S*–*S*–HSA; 1/10,000 dilution for CP–*S*–*S*–KLH) with CP–disulphide, (IC_{50} for the CP–*S*–*S*–HSA antiserum = 63 μg/ml; IC_{50} for the CP–*S*–*S*–KLH antiserum = 22 μg/ml) thus confirming again the specificity of the anti-CP IgG activity.

Contact sensitivity to captopril and penicillin G in the guinea pig

The results for the guinea pig maximization test are shown in Table 1. When challenged with CP,

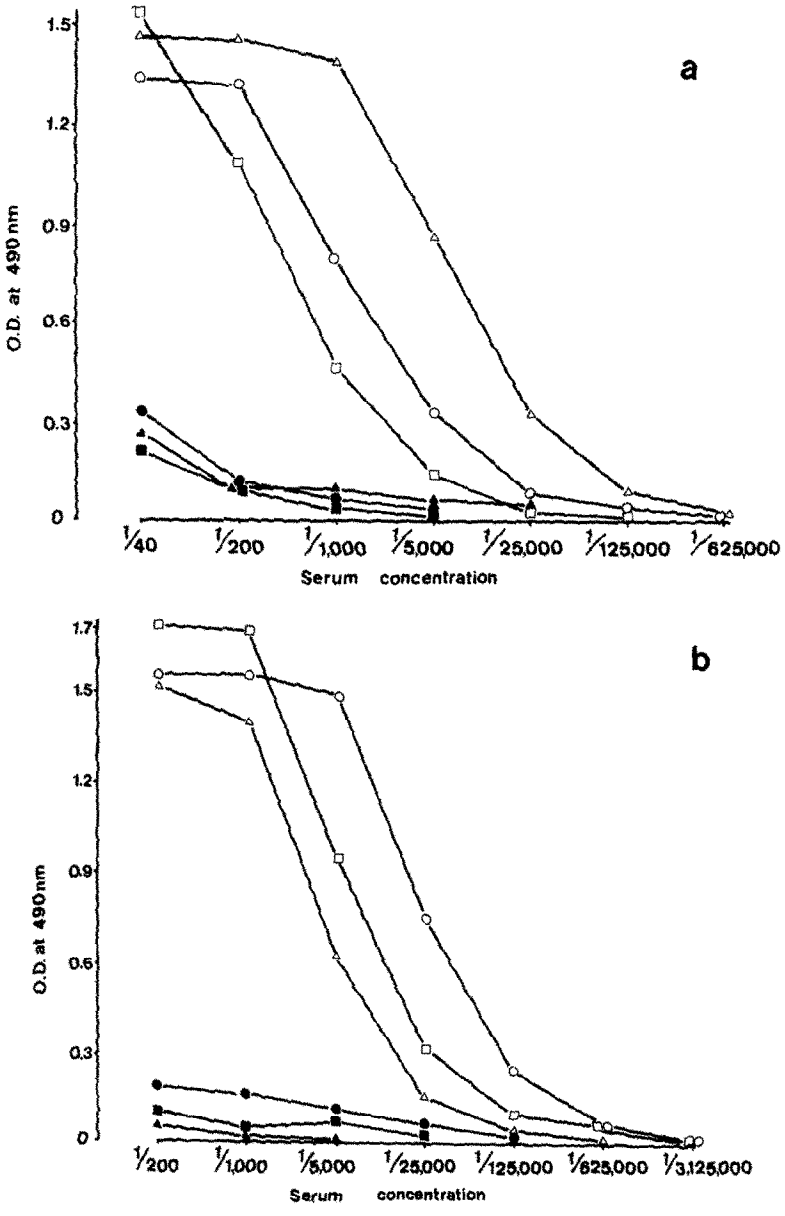

Fig. 3. CP-specific IgG antibody activity in the sera of rabbits immunized with (a) the CP–*S*–*S*–HSA conjugate and (b) the CP–*S*–*S*–KLH conjugate, measured by ELISA. Each type of symbol (△, ○, □) represents the individual animal in each group. The closed symbols represent the pre-immunization serum of the respective animals.

four out of the total seven guinea pigs showed an erythematous reaction (reddening of the skin) which persisted for 24 hr, while the rest showed mild redness on the challenged area. This reaction to CP was similar to the positive control group that had been sensitized and challenged with penicillin G, a known skin sensitizer in both the guinea pig [18] and in man [19]. The control group (sensitized and challenged with the vehicle only) showed a sensitization rate of 0/8.

DISCUSSION

According to the "hapten hypothesis", drugs or low mol. wt chemicals must become covalently bound to autologous macromolecules to induce hypersensitivity [9]. The role of chemically reactive metabolites of drugs in immunotoxicity is yet to be established. However, drugs such as penicillin and practolol are metabolised to chemically reactive derivatives which bind to proteins, thus enabling the drugs to act as haptens *in vivo* [20, 21]. Other drugs which are not metabolised to chemically reactive species, but rather contain reactive group(s) in their molecular structure (e.g. sulphydryl group of penicillamine) may also have the potential to function as haptens. The purpose of this investigation was to determine whether protein conjugates containing

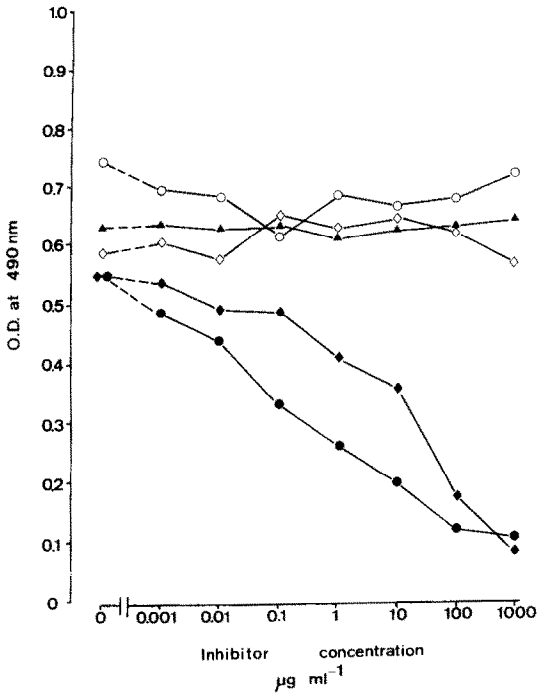

Fig. 4. Inhibition of captopril (CP)-specific IgG activity by preincubation of rabbit antiserum (obtained after the third injection of CP–*S*–*S*–HSA; 1/5000 dilution) with various concentrations of disulphide-linked drug-protein conjugates and carrier proteins: CP–*S*–*S*–OVA (●), penicillamine–*S*–*S*–OVA (▲), OVA (○), CP–*S*–*S*–KLH (◆), and KLH (◇). The CP-specific IgG activity was measured by ELISA.

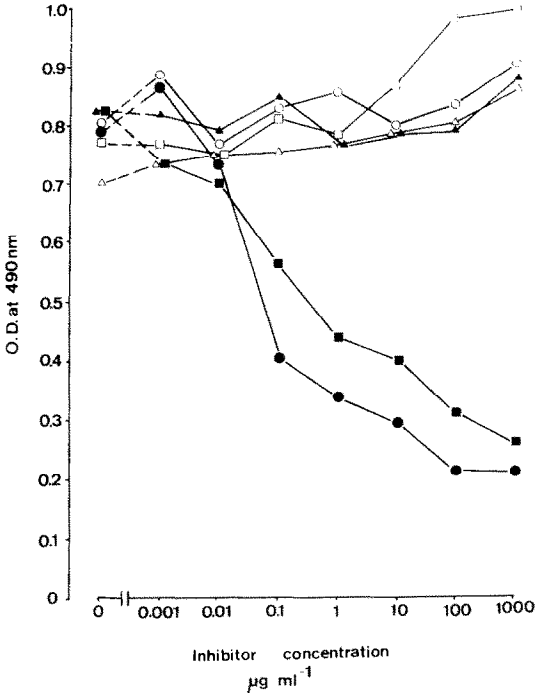

Fig. 5. Inhibition of CP-specific IgG activity by preincubation of rabbit antiserum (obtained after the third injection of CP–*S*–*S*–KLH, 1/10000 dilution) with various concentrations of disulphide-linked drug–protein conjugates and carrier proteins: CP–*S*–*S*–OVA (●), penicillamine–*S*–*S*–OVA (▲), OVA (○), CP–*S*–*S*–HSA (■), penicillamine–*S*–*S*–HSA (△) and HSA (□). The CP-specific IgG activity was measured by ELISA.

CP, linked via a disulphide bond, are sufficiently stable *in vivo* to allow recognition of CP as a hapten by the immune system. These studies were prompted by previous metabolic studies which revealed that although CP readily forms conjugates with autologous proteins, such conjugates are metabolically labile [10, 12].

For immunochemical studies, CP was linked to two foreign proteins, HSA and KLH, via disulphide bonds using mild oxidation conditions. These heterologous proteins were used for two reasons: firstly to optimise the conditions for an immune response; secondly, to monitor immune recognition (and immune interaction) of the macromolecular carrier. The problems associated with investigations of drug hypersensitivity have been the lack of suitable methods and model compounds. A major obstacle in the detection and quantification of antibodies to drug-haptens is that the chemical nature of the antigenic determinant is specific, but difficult to predict. We therefore used a battery of immunochemical test systems, employing a range of hapten–protein conjugates, to probe for antibody response.

Immunoprecipitation has previously been used for

Table 1. The maximization test for captopril and penicillin G in the guinea pig

	Induction		Challenge		
Treatment	Intradermal injection (0.2 ml in adjuvant in water)	Topical application (0.4 ml in 40% Tween 80 in water)	Topical application (0.4 ml in 40% Tween 80)	Degree of sensitization for each animal	Sensitization rate
Captopril	5% w/v	10% w/v	10% w/v	1, 1, 1, 2, 2, 2, 2	7/7 (100%)
Penicillin G	3% w/v	5% w/v	10% w/v	1, 1, 1, 1, 2, 2, 2, 2	8/8 (100%)

The criteria for an immune response are redness and swelling of the skin, scored on a three-point scale: 0 = no reaction, 1 = mild redness and 2 = intense and swelling. To avoid any bias in judgement, the assessment procedure was designed in such a way that the assessors examined the animals at random, without knowing the type of treatment the animals had received.

the detection of antibodies directed against penicillamine in rabbits immunized with a disulphide-linked penicillamine–HSA conjugate [22]. Using this technique, we were able to demonstrate a clear antibody response to HSA in rabbits immunized with a CP–*S*–*S*–HSA conjugate, but it was not possible to detect a response to CP, using a variety of CP–protein conjugates as the test antigen. However, using the same technique, antibodies were detected against CP when rabbits were immunized with CP linked to HSA via an amide bond which is both chemically and metabolically [14] more stable than a disulphide bond [10, 12]. Interestingly, hapten inhibition studies revealed that the antigenic determinant contained CP and a lysine residue from the protein carrier. Furthermore, the degree of chemical substitution (epitope density = 23) of CP on the amide-linked CP–HSA conjugate was sufficient to mask the natural antigenic determinants on HSA, hence there was no immunoprecipitate line formed between the antiserum and HSA.

Immunoprecipitation is a relatively insensitive technique and can therefore only be used to detect a hyperimmune response. We therefore went on to explore more sensitive radioimmunoassay and ELISA methods. By using a radioimmunoassay method, we were unable to detect antibodies directed against CP in rabbits immunized with disulphide-linked CP–protein conjugates (CP–*S*–*S*–HSA and CP–*S*–*S*–KLH). The radioligands employed (captopril disulphide and *S*-acetyl-captopril) were chemical derivatives of CP. CP itself could not be used as a radioligand because of its direct chemical reactivity with proteins which contain disulphide bonds (e.g. immunoglobulins) [10, 11, 23].

In the ELISA for circulating anti-CP antibodies in rabbits immunized with the disulphide-linked CP–protein conjugates (CP–*S*–*S*–HSA AND CP–*S*–*S*–KLH), a CP–*S*–*S*–OVA conjugate (epitope density 7:1) was selected as the immobilised ligand because (a) parallel studies showed little or no anti-OVA activity; (b) in view of the results obtained with the amide-linked CP–protein conjugate, it was reasoned that part of the antigenic determinant might be derived from the protein. A detectable response (titre range: 1/459–1/6474) was observed in rabbits immunized with CP–*S*–*S*–HSA, but a somewhat greater response (titre: 1/3103–1/19367) was measured following immunization with the disulphide-linked CP–*S*–*S*–KLH conjugate. This difference probably reflects differences in the immunogenicity of these proteins [24], rather than a difference in the metabolic stability of the drug–protein conjugates. The binding of antibodies raised against CP–*S*–*S*–KLH, to CP–*S*–*S*–OVA could be inhibited by a third conjugate, CP–*S*–*S*–HSA, but not by unconjugated HSA, nor penicillamine–*S*–*S*–HSA, demonstrating specificity of the antiserum for CP. These results therefore show that disulphide-linked CP–protein conjugates are sufficiently stable, *in vivo*, to promote a specific anti-hapten B-lymphocyte response.

To further investigate the immunogenic potential of CP, we studied the capacity of CP to induce contact sensitivity, essentially a measure of cell-mediated immunity, by the guinea pig maximization test. In line with other compounds with free thiol groups [25], captopril produced a specific and positive response in sensitized animals. The time course and nature of the response were indistinguishable from that observed for penicillin G, which is a known contact sensitizer in man [19] and in the guinea pig [18]. It was not possible to define the nature of the antigenic determinant involved in the contact sensitivity to CP in this study. However, we assume in this test system that captopril was linked to dermal proteins via a disulphide bond, particularly as the latter are rich in cysteine/cystine residues [26]. It should be noted that the guinea pig maximization test merely gives an indication of the potential of an agent to induce contact sensitivity, and does not necessarily imply that an agent which causes an allergic skin reaction when applied topically would also cause the same effect when given by other routes of administration.

Taken collectively, these data indicate that a disulphide bond is sufficiently stable to carry CP through the various biochemical and cellular processes, including handling by macrophages [27], which lead ultimately to recognition by B and T lymphocytes in humoral and cell-mediated immunity respectively. However, it cannot be concluded from this study that CP disulphide-linked to autologous proteins rather than heterologous proteins, will necessarily be immunogenic. Furthermore, Freund's Complete Adjuvant was used in conjunction with heterologous carrier proteins in this study, in order to produce extremely sensitive model test systems. Freund's Complete Adjuvant is an immunostimulant which has a number of effects on the immune system, none of which are well defined [28]. Nevertheless, having established the chemical nature of the potential haptenic form of captopril *in vivo* and having developed a specific and sensitive immunochemical technique (based on ELISA), we are now in a position to determine whether or not patients taking CP produce antibodies directed against the drug.

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