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The role of metabolism in the immunogenicity of drugs: Production of antibodies to a horseradish peroxidase generated conjugate paracetamol

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SUMMARY

The allergic response to small chemically inert molecules in thought to require their enzymatic conversion to reactive metabolites which are then endowed with the capacity to bind covalently to host proteins and produce immunogenic hapten-carrier conjugates.

In contrast to previous studies in which hapten-carrier conjugates have been generated following chemical modification of drugs we have examined the immunogenicity of paracetamol following direct conjugation to carrier proteins with horseradish peroxidase (HRP).

Highly substituted conjugates of paracetamol with keyhole limpet haemocyanin (KLH) or bovine serum albumin (BSA) were generated using HRP. The KLH conjugate was used to immunize Balb/C mice. IgM and IgG (predominantly IgG_1) responses were observed and shown by enzyme-linked immunosorbent assay (ELISA) to be hapten-specific. Manipulations of HRP levels permitted substitution of KLH to varying extents with paracetamol. Such conjugates were tested for their ability to induce a hapten-specific immune response. It was determined that substitution of 1 mol of KLH with 700 mol of paracetamol was sufficient to generate an anti-hapten response. These data suggest a mechanism by which protein-non-reactive drugs may be rendered immunogenic and provide a method for demonstrating the presence of serum antibodies reactive with drug metabolites.

Keywords drug allergy parcetamol horseradish peroxidase metabolism

INTRODUCTION

There is now considerable evidence that a variety of drugs may cause hypersensitivity reactions in certain susceptible individuals (Carr, 1954; Van Arsdel, 1983). The mechanism through which small haptenic drugs invoke tissue-damaging immune reactions in susceptible recipients is, however, unclear. The importance of stable bonds between small molecules and proteins in the manifestation of immune responses was first recognized by Landsteiner (1933). Such conjugates permit the induction of antibody responses to otherwise non-immunogenic haptens (Mitchison, 1971). Davies (1958) proposed that the development of allergic responses to small ($< 10^3$ daltons) protein-non-reactive drugs would necessitate their conjugation to host macromolecules *in vivo*. There is no direct evidence to suggest that such a mechanism exists and the failure to detect drug-reactive antibodies in

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the sera of patients who exhibit clinical manifestations of adverse reactions is apparently incompatible with the immunogenicity of such drugs (Kappus & Remmer, 1975; Van Arsdel, 1983). It has been suggested, however, that drug-induced reactions are associated with antibody production, but that such antibodies are directed against a protein-reactive metabolite of the drug rather than the drug itself (Levine, 1966; Eisner & Shahidi, 1972; Parker, 1980). A corollary of this is that chemically formed drug-protein conjugates prepared *in vitro* are unlikely to provide a suitable substrate for the demonstration of antibodies reactive with electrophilic drug intermediates generated *in vivo* (Davies, 1958; Eisner & Shahidi, 1972).

In order to further clarify the role of metabolism in the induction of antibody responses to small molecules we have examined the immunogenicity of paracetamol, a protein non-reactive drug of mol.wt 151.1, following its conjugation to heterologous carrier proteins under conditions analogous to those which are likely to be of relevance *in vivo*. We demonstrate that immunization of mice with such 'metabolically' formed conjugates induces antibodies reactive with protein-bound paracetamol but not with the drug in its native form.

MATERIALS AND METHODS

Synthesis of paracetamol/protein conjugate

Materials. Horse radish peroxidase (HRP) type VI, paracetamol, bovine serum albumin (BSA) and hydrogen peroxide were purchased from Sigma Chemical Company (St Louis, Missouri, USA) radiolabelled [^{3}H]paracetamol (s.a. = 11 Ci/mmol) was obtained from New England Nuclear (Boston, Massachusetts, USA) and keyhole limpet haemocyanin (KLH) from Calbiochem (La Jolla, California, USA).

Method. Standard 3-ml reaction mixtures contained HRP (6 U/ml), H_2O_2 (50 mmol), 10 μmol paracetamol spiked with 500 c.p.m. per nmol [^{3}H]paracetamol, and either bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH), 6 mg in 0·05 M sodium phosphate buffer (pH 7·4). The order of addition was buffer (0·5 ml), BSA or KLH (1 ml, 6 mg/ml) H_2O_2 (50 μl) and HRP (1 ml, 5 U/ml). Reaction mixtures were dialysed extensively against several changes of physiological saline over a period of 5 days at 4°C. The percentage of [^{3}H]paracetamol bound to the high-mol.wt material was calculated and used to define the degree of substitution of paracetamol with protein. Two control reactions were incubated in parallel, lacking either HRP or paracetamol, the latter being used for control immunizations. Conjugates of varying levels of substitution were prepared by alteration of the concentration of HRP in the reaction mixture, the degree of substitution being determined using [^{3}H]paracetamol.

Immunization with paracetamol-KLH. Male Balb/c mice (20–30 g) were immunized with 100 μg paracetamol-KLH in Freund's complete adjuvant (FCA) (Difco Laboratories, Detroit, Michigan, USA), administered at four subcutaneous sites and intraperitoneally. Fourteen days later the animals received a challenge employing the same regime but with incomplete adjuvant (FIA). On days 28–31 the animals received four consecutive daily immunizations of 100 μg conjugate in physiological saline intraperitoneally; they were sacrificed on day 32 and blood withdrawn by cardiac puncture. Serum was separated by centrifugation and stored at −20°C.

Indirect ELISA for IgG antibodies to KLH paracetamol

- 200 μl of an optimal concentration of BSA-paracetamol (145 mM) in coating buffer (Na_2CO_3 (15 mM), $NaHCO_3$ (35 mM) and NaN_3 (3 mM) pH 9·6) was added to the wells of a microtitre plate. After 2 h at 20°C the plates were washed three times with phosphate-buffered saline containing 0·5% Tween 20 (pH 7·4), (PBS-T).
- To minimize non-specific binding 2% BSA in coating buffer was added to all the wells in 200 μl volumes.
- Serial two-fold dilutions of serum in PBS-T were applied to the plate in 150 μl aliquots; the highest concentration being 1:100. Following 2 h incubation at 20°C the plates were washed three times with PBS-T.
- Peroxidase-labelled rabbit anti-mouse IgG (1:4000) (Miles Laboratories, Slough, England) was added in 200 μl volumes and incubated for 2 h at 20°C.
- After washing, 200 μl of substrate (O-phenylene diamine (4·42 mM) and urea hydrogen

peroxidase (2·0 mM) (Sigma Chemical Company) was added and left for 30 min in the dark. Substrate oxidation was terminated with 50 μl 0·5 M citric acid. The optical density of each well was determined at wavelength 450 nm with a Multiscan spectrophotometer (Flow Laboratories, Scotland).

Indirect ELISA for IgM antibodies to KLH-paracetamol. The same procedures used for the detection of IgG to KLH-paracetamol were applied except that step (4) involved a 2-h incubation at 20°C with 200 μl/well rabbit anti mouse IgM (1:4000) (Miles Laboratories). After washing a 1:4000 dilution of peroxidase-labelled goat anti-rabbit IgG (Miles Laboratories) was added in 200 μl volumes and incubated for 2 h at 20°C.

Indirect ELISA for the subclass of IgG to KLH-paracetamol. The procedure was identical to that used in the measurement of whole IgG to KLH-paracetamol except that step (4) first involved a 2-h incubation at 20°C with a 1:4000 dilution of the appropriate rabbit anti-mouse IgG subclass antiserum (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3) (Miles Laboratories). After washing 1:4000 dilution of goat anti-rabbit IgG enzyme linked (HRP) was added, (200 μl per well), and incubated at 20°C for 2 h.

Inhibition ELISA to demonstrate the specificity of the response to paracetamol. The procedure was identical to that used in the indirect ELISA for IgG to KLH-paracetamol, except that step (3) involved the addition of various concentrations of inhibitor to the reaction mixture in 100 μl volumes. Serum samples (100 μl/well) were then applied to the plate at a dilution of 1:200 in PBS-T giving a final dilution of 1:400.

Percent inhibition of antibody binding was calculated as

$$\frac{Ao - Ai}{Ao} \times 100$$

where A was the absorbance in the absence (Ao) or presence (Ai) of inhibitor.

Radioimmunoassay for antibodies to the native paracetamol molecule. Serum shown by ELISA to be reactive with KLH paracetamol was diluted with phosphate-buffered saline, pH 7·4, to provide a 1:5 dilution. To determine the serum-binding capacity of the antiserum, 0·5 ml of the 1:5 dilution was incubated with 38 ng [^{3}H]paracetamol for 2 h at 37°C and for a further 15 min at 4°C. The antibody-bound [^{3}H]paracetamol was separated from free hapten by the addition of 0·5 ml saturated ammonium sulphate (SAS) as described by Farr (1958). After two washings with 50% SAS, the precipitate was dissolved in 0·5 ml physiological saline. The contents of the tube were transferred to a vial and counted on an LKB Minibeta Scintillation Counter. Sera obtained from animals immunized with HRP-treated KLH alone and from animals immunized with KLH-imipramine were examined in parallel.

RESULTS

Peroxidase-mediated binding of a metabolite of paracetamol to BSA and KLH

Incubation of [^{3}H]paracetamol with BSA and KLH in the presence of HRP (2·0 u/ml) and hydrogen peroxide for 30 min at 20°C, resulted in the formation of a metabilite which bound irreversibly to

Table 1. Peroxidase-mediated binding of a metabolite of paracetamol to BSA and KLH

	Bound ^{3}H-paracetamol to high-mol.wt material (%)	Paracetamol bound protein (nmol/mg)	Molar substitution ratio
BSA			
Test	19·25	10·89	21·90
Control	0·01	0·08	0·01
KLH			
Test	21·51	182.33	1075·00
Control	1·04	9·53	52·00

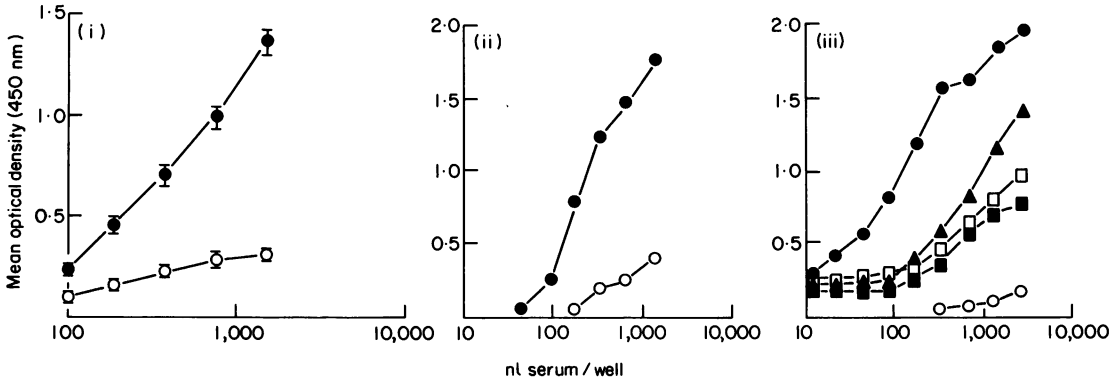

Fig. 1. Indirect analysis by ELISA of the immune response to paracetamol-KLH. (i) IgG response to paracetamol-KLH (●) and HRP treated KLH control (○). Results are expressed as means ± s.e. (ii) IgM response to paracetamol-KLH (●) and HRP-treated KLH (○). (iii) IgG subclass response to paracetamol-KLH. IgG (●); IgG_{2a} (□); IgG_{2b} (■); IgG_3 (▲).

protein as demonstrated by extensive dialysis. Table 1 shows the percentage of BSA- and KLH-bound tritiated paracetamol respectively. With respect to BSA, 19·25% tritiated paracetamol remained associated with protein after extensive dialysis. The demonstration that in the absence of HRP only 0·01% of the [^{3}H]paracetamol was bound confirms the requirement for enzyme in the production of stable conjugates. Similarly, 21·5% of the tritiated paracetamol was found to be associated with KLH after dialysis compared with only 1·04% in the absence of HRP. The reason for the elevation of the control value for KLH over BSA, 1·04 compared with 0·01 is unclear and cannot be explained solely on the basis of the differing molecular weights of the proteins.

The values obtained for the degree of substitution of paracetamol with KLH (mol. wt $= 3 \times 10^6$) and BSA (mol. wt $= 6{\cdot}9 \times 10^4$), 1075 and 21·9 respectively are representative of immunogenic conjugates. The KLH-paracetamol conjugate was used for immunizations.

Indirect ELISA for the detection of IgG and IgM to KLH-paracetamol

Sera obtained from 30 mice immunized with KLH-paracetamol (1075) and 30 control mice immunized with peroxidase-treated KLH were assayed for the presence of IgG antibodies reacting with BSA-conjugated paracetamol by indirect ELISA. Clear differences between test and control values were observed over a range of dilutions (Fig. 1(i)).

Although the values obtained for IgM (Fig. 1(ii)) were comparable with those recorded for IgG it is important to emphasize that IgM antibodies were measured using an indirect ELISA incorporating an intermediate antibody (rabbit anti-mouse IgM) which serves to amplify the response. Clear comparisons are therefore unavailable although it is likely that IgG represents the major component of the anti-KLH paracetamol response. The predominant subclass of IgG involved in the response to KLH-paracetamol was found to be IgG_1, the trend being $IgG_1 > IgG_3 > IgG_{2b} > IgG_{2a}$ (Fig. 1(iii)).

Optimal epitope density for the production of antibodies to KLH-paracetamol

Hapten-protein conjugates differing in the degree of substitution of paracetamol were obtained following variation of HRP concentration added to the reaction mixture. To avoid any disruption of the quaternary structure of the protein carrier by enzymatic degradation the maximum HRP concentration employed was 2·0 U/ml. Table 2 shows the optical density at wavelength 450 nm for a 1:100 dilution of pooled serum taken from mice immunized with a range of paracetamol-substituted KLH-conjugates. Molar substitution ratios in excess of 700 provided good immunogens; below this level the ability of conjugates to induce a detectable hapten-specific immune-response was equivocal.

Specificity of the IgG response to KLH-paracetamol

(i) *Inhibition ELISA studies.* The reactivity of pooled anti-KLH-paracetamol sera with BSA-paracetamol could not be inhibited by free paracetamol (Fig. 2). This suggested that the antibody formed in response to the KLH-paracetamol conjugate was specific for a metabolite of paracetamol. This hypothesis was validated by the observation that KLH-paracetamol, but not

Table 2. Influence of epitope density on the production of antibodies to KLH-paracetamol. Antibody concentration was measured by ELISA using a BSA-paracetamol substrate

Conjugate	HRP (U/ml)	Epitope density (molar substitution ratio)	Mean optical density at 450 nm for 1:100 dilution of pooled serum
1	2·0	1076	1·48
2	1·0	705	0·79
3	0·5	544	0·45
4	0·25	275	0·45
Control	2·0	HRP treated KLH	0·32

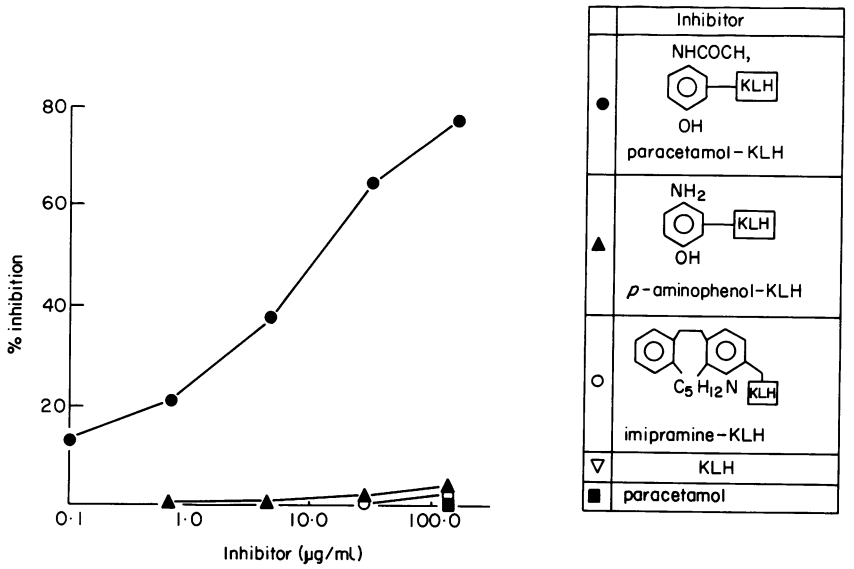

Fig. 2. Inhibition ELISA to demonstrate the specificity of the anti-paracetamol-KLH response for paracetamol. The various inhibitors indicated were incubated with antiserum on microtitre plates coated with BSA-paracetamol. The final dilution of the antiserum was 1:400.

HRP-treated KLH caused a dose-dependent inhibition of binding (Fig. 2). Significant inhibition was observed at a concentration of 10 ng/ml KLH-paracetamol with 78% inhibition occurring at the highest inhibitor concentration employed (168 μg/ml) (Fig. 2). It should be stressed that although the concentration of KLH is relatively high the structure actually involved in the competitive inhibition, the metabolite of paracetamol and its 'link' with the KLH-carrier will be of extremely low concentration (approximately 10 nmol paracetamol bound to KLH at the highest concentration of inhibitor conjugate).

The specificity of the response for a metabolite of paracetamol was further illustrated by the demonstration that other drug-KLH-conjugates (KLH-imipramine and KLH-*p*-aminophenol) failed to inhibit binding to BSA-paracetamol (Fig. 2).

(ii) *Radioimmunoassay for antibodies to the unchanged drug.* The ELISA system employed was designed to quantitate antibodies reactive with a protein-bound metabolite of paracetamol and was thus inappropriate for the demonstration of antibodies specific for the native drug. Radioimmunoassay incorporating tritiated paracetamol confirmed the absence of antibodies specific for the native drug.

DISCUSSION

Although the mechanism of drug allergy is largely unresolved it is widely accepted that metabolism plays a critical role in converting the native drug into a reactive species possessing the capacity to conjugate with host macromolecules (Parker, 1980; Dupuis, 1972).

Similarly, it has been demonstrated that carcinogens such as benzypyrene (Gelboin, 1969) and vinyl chloride (Green & Hathway, 1978) are converted into potent alkylating molecules which have the capacity to bind to host DNA. What is clear from such studies is that the stable compound is extensively metabolized and the reactive group that binds to host macromolecules differs markedly from the parent compound. The metabolite responsible for the induction of allergy may also be structurally dissimilar to the original drug and screening methods employing the native compound may fail to detect immune reactivity.

Paracetamol was chosen for investigation since previous studies demonstrate that a reactive metabolite (acetamidoquinone) can induce liver necrosis following irreversible binding to hepatic protein (Potter *et al.*, 1973). The *in vitro* activation of paracetamol thus provides a valuable model for examining the potential of metabolically formed conjugates to induce immune responses.

The use of a HRP/hydrogen peroxidase (H_2O_2) system to generate metabolites of paracetamol was employed for the following reasons. First, it is clear that the liver microsomal oxygenase can function as a peroxidase (Rahimtula & O'Brien, 1974). Second, Nelson *et al.* (1981) described the peroxidase-mediated formation of a metabilite of paracetamol and demonstrated that the binding paralleled that mediated by the cytochrome P-450 system. Third, by experimental manipulation conjugates could be generated with degrees of substitution comparable to chemical modification methods and finally, well-defined carriers such as BSA and KLH can be employed which permit more exacting specificity analysis.

The observed correlation between the *in vitro* and *in vivo* binding of paracetamol to microsomal protein (Potter *et al.*, 1973) and the ability of HRP-metabolized paracetamol to form a hapten-carrier conjugate prompted investigation of the potential of the liver monoxygenase system similarly to render the drug immunogenic *in vitro*. Even with the optimum conditions of phenobarbitone-induced and glutathione-depleted rat microsomes the levels of binding observed were insufficient to render the drug immunogenic; implying that under normal circumstances paracetamol should not be immunogenic *in vivo*. Indeed, it is clear from the epitope density study, employing HRP-generated conjugates, that extensive substitution of protein with paracetamol is necessary to render the drug immunogenic (Table 2). One would not expect, therefore, that the dose of paracetamol administered therapeutically (900–1800 mg/day in adults) would produce a sufficiently high concentration of the protein reactive metabolite to render the drug immunogenic *in vivo*. The fact that liver necrosis, induced by covalent binding of the paracetamol metabolite to hepatic protein (Jollow *et al.*, 1973), does not occur with single doses less than 10 g (Drayer, 1982) supports this view. This may serve to explain the relatively low incidence of allergy among individuals receiving the drug.

In conclusion, we have demonstrated the potential of metabolically formed drug-protein conjugates generated *in vitro* to induce an immune response in the mouse. Furthermore, such conjugates represent potentially useful substrates for the analysis of drug-reactive antibodies.

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