Biochemical Pharmacology, Vol. 36, No. 5, pp. 591–599, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

DRUG–PROTEIN CONJUGATES—XI

DISPOSITION AND IMMUNOGENICITY OF DINITROFLUOROBENZENE, A MODEL COMPOUND FOR THE INVESTIGATION OF DRUGS AS HAPTENS

B. KEVIN PARK,* MALCOLM D. TINGLE, PETER S. GRABOWSKI, JOHN W. COLEMAN and NEIL R. KITTERINGHAM

Department of Pharmacology and Therapeutics, The University of Liverpool, Liverpool L69 3BX, U.K.

(*Received* 19 *June* 1986; *accepted* 1 *August* 1986)

Abstract—The conjugation of drugs to autologous proteins is thought to be a key step in the hapten mechanism of drug hypersensitivity. We have studied the mild arylating agent dinitrofluorobenzene (DNP-F) as a model compound with which to investigate the relationship between the disposition and immunogenicity of drug haptens in two species, the rat and rabbit. Intramuscular administration of DNP-F (0.027–27 μmol/kg/day) for 4 days to male Wistar rats produced a dose-dependent (ED_{50} 2.7 μmol/kg) IgG anti-DNP antibody response, measured by enzyme-linked immunosorbent assay. Subsequent monthly administrations (for 4 days) increased both the frequency and titre of antibody response. Intravenous administration of [^{3}H]DNP-F (0.27 or 2.7 μmol/kg) for 9 days to male New Zealand White rabbits produced an IgG and IgM anti-DNP response in all animals from day 9 onwards. Formation of circulating (serum) DNP–protein conjugates was determined by radiometric analysis, and found to reach steady state (0.12–0.17% dose/ml) between days 6 and 8 and decline with a half-life of 7.4 days. The immunogenicity of fully characterized haptenated, autologous proteins was investigated in further experiments in which dinitrophenylated serum protein conjugates (DNP–RSP) and albumin conjugates (DNP–RSA) were prepared *ex vivo* and then administered (50 mg/kg; i.v.) to the rabbit from which the protein had been obtained. The plasma clearance and immunogenicity of DNP–RSA conjugates was dependent on epitope density. Anti-DNP antibodies were detected after administration of an RSA–DNP_{15} conjugate but not after either RSA–$DNP_{0.5}$ or RSA–DNP_5. Plasma concentrations of RSA–DNP_{15} conjugate declined slowly initially, but then fell rapidly between days 8 and 10. The plasma clearance of DNP–RSP conjugates showed a dependence on epitope density from day 1 onwards and anti-DNP antibodies were detectable after administration of all conjugates investigated (range of epitope densities 0.5–30 DNP/albumin equivalent). Thus conjugates derived from proteins other than albumin are likely to be the effective immunogens, for the model hapten DNP. These studies show that DNP-F is a useful model compound in studies of the disposition and immunogenicity of drugs acting as haptens, and may therefore be used as a positive control in experiments designed to assess the potential immunogenicity of drugs and other xenobiotics.

Many adverse drug reactions are ascribed an immunological mechanism, but the aetiology of drug hypersensitivity is poorly understood [1, 2]. According to the hapten hypothesis, it is thought that the drug, or a chemically reactive metabolite, becomes covalently bound to an endogenous macromolecule (e.g. circulating protein) and that the resulting conjugate may then function as an immunogen or antigen. Combination of antigen with either specific antibody or effector T-cells may lead to a hypersensitivity reaction.

This hypothesis is based on classical immunochemical studies which have shown that the ability of low molecular weight compounds to induce an antibody response is a direct function of their chemical reactivity with nucleophilic groups on proteins or other macromolecules [3–6]. However, co-administration of an adjuvant and conjugation to a foreign protein are nearly always used to intensify the immune response. Less information is available concerning the immunogenicity of either free drugs or haptens conjugated to autologous proteins [7, 8], especially after administration via routes normally used for drugs.

The object of this work was to investigate dinitrofluorobenzene (DNP-F†) as a model compound with which to assess the disposition and immunogenicity of haptens derived from drugs, or their metabolites, and autologous protein. DNP-F was selected for this purpose because (1) it binds covalently to proteins under mild non-denaturing conditions and (2) our previous studies have shown that it is useful as a model chemically reactive metabolite for the investigation of hapten–protein conjugation *in vivo* [9, 10].

MATERIALS AND METHODS

2,4-[3,5-^{3}H]Dinitrofluorobenzene ([^{3}H]DNP-F), sp. act. 16.6 Ci/mmol, was obtained from Amersham

* Author to whom correspondence should be addressed.

† Abbreviations: DNP-F, dinitrofluorobenzene; ELISA, enzyme-linked immunosorbent assay; RSA, rabbit serum albumin; RSP, rabbit serum protein; BSA, bovine serum albumin; HSA, human serum albumin; OVA, ovalbumin.

International (Amersham, U.K.). Bovine serum albumin (BSA), ovalbumin (OVA), unlabelled DNP-F and lysine were obtained from Sigma Chemical Ltd. (London, U.K.). All other reagents were from BDH Ltd. (Poole, U.K.). Microtest III enzyme-linked immunosorbent assay (ELISA) plates were from Becton Dickinson (Oxford, U.K.). Goat anti-rabbit IgG and IgM, and horseradish peroxidase-labelled rabbit anti-goat IgG were obtained from Miles Laboratories (Slough, U.K.). Rabbit anti-rat IgG, and horseradish peroxidase-labelled goat anti-rabbit IgG were obtained from Nordic Immunological Laboratories (Maidenhead, U.K.). Male New Zealand White rabbits were obtained from Bantin and Kingman (Hull, U.K.).

Disposition and immunogenicity of [^{3}H]*DNP-F in the rabbit.* [^{3}H]DNP-F was diluted with unlabelled DNP-F to a specific activity of either 3 μCi/mg or 30 μCi/mg in redistilled toluene, and stored at 4° in a tightly sealed container until used. An aliquot (0.24 ml) of [^{3}H]DNP-F was taken and the toluene removed by evaporation under a gentle stream of nitrogen. The DNP-F was redissolved in 1.2 ml polyethylene glycol 200 immediately prior to injection. Groups of four male New Zealand White rabbits weighing 2.4 ± 0.2 kg received [^{3}H]DNP-F (either 2.7 or 0.27 μmol/kg) intravenously in polyethylene glycol 200 (0.1 ml/kg) daily for 9 days. Blood (1–2 ml) was collected into heparinized containers 24 hr after administration of [^{3}H]DNP-F, prior to re-administration, during the 9-day treatment period, and regularly over the following 16 days. On day 9, a sample of blood was collected into a heparinized container and mixed immediately with lysine (1 mg/ml). Plasma was separated after centrifugation, and duplicate aliquots (200 μl) were mixed with scintillant (Scintillator 299) and assayed for radioactivity in a Beckman liquid scintillation spectrometer. The quantity of radioactivity irreversibly bound to plasma proteins was assessed in the samples mixed with lysine. The elimination half-life of radioactivity from day 9 to day 25 was calculated by log-linear regression for individual rabbits.

The IgG and IgM anti-DNP antibody responses were determined by ELISA as described below.

Immune response to DNP-F in the rat. Groups (N = 4–6) of male Wistar rats (250–300 g) received DNP-F (0.027–27 μmol/kg/day) for 4 days. The treatment was repeated 4 weeks and 8 weeks after the initial set of injections. Blood samples were obtained 14 days after each set of injections from the tail vein, allowed to stand overnight at room temperature, centrifuged (2000 *g*) and serum stored (−56°) until assayed for anti-DNP antibodies by ELISA as described below.

Preparation of dinitrophenylated bovine serum albumin (BSA-DNP). Bovine serum albumin (4 g) was dissolved in 40 ml 0.1 M phosphate buffer, pH 7.4. [^{3}H]DNP-F (6.4 mg, 240 μCi) was added to the protein solution in 0.5 ml dry dioxan, and incubated at 37° overnight in the dark in a shaking water bath. After mixing with lysine (20 mg) for 1 hr, the conjugate was chromatographed in aliquots (5 ml) at 4° on a column of Sephadex G25 (bed volume, 100 ml) in distilled water, and the fractions containing protein were pooled, frozen at −70° and lyophilized. Prior to administration the conjugates were filtered (0.2 μm pore size) and centrifuged at 105,000 *g* to remove any insoluble protein. The mean number of DNP groups per molecule of protein was determined radiometrically to be 0.5.

Preparation of dinitrophenylated rabbit serum albumin (RSA–DNP). Blood (20 ml) was collected from the marginal ear vein of male New Zealand White rabbits (2–2.5 kg) and allowed to clot overnight at room temperature. Serum was decanted after centrifugation (2000 *g*) for 15 min and processed immediately or stored at −20°.

Rabbit serum albumin was isolated using a modification of a method used previously [11]. Cibacron Blue–agarose (20 ml wet volume) was equilibrated with Tris–HCl buffer (0.05 M, pH 8.0) containing 0.05 M NaCl, in a round-bottomed, stoppered centrifuge tube. Serum (5 ml) was diluted with the same buffer (15 ml) and gently mixed with the Cibacron Blue–agarose at room temperature for 30 min. After centrifugation (1000 *g*) for 15 min, the supernatant was removed and discarded. The Cibacron Blue–agarose was gently rinsed three times with equilibration buffer (15 ml) by alternate suspension and centrifugation, discarding the supernatants. Rabbit serum albumin was eluted from the Cibacron Blue–agarose by suspension in equilibration buffer (20 ml) containing 0.2 M NaSCN, for 30 min at room temperature. The supernatant containing the rabbit serum albumin was carefully removed after centrifugation. The Cibacron Blue–agarose was rinsed a further two times with the elution buffer (15 ml) and the supernatants pooled, filtered through a Sartorius sterile filter (0.2 μm pore size) and dialysed for three days against six changes of distilled water (5 l) at 4°. The dialysed rabbit serum albumin was frozen at −70° and lyophilized. A yield of 300–350 mg albumin was obtained from 10 ml serum.

Rabbit serum albumin (250 mg) was dissolved in 10 ml 0.1 M phosphate buffer, pH 7.4. DNP-F (0.4, 5 or 13.5 mg) and [^{3}H]DNP-F (50 μCi) was added to the protein solution in 0.5 ml dry dioxan, and incubated overnight at 37° in the dark, in a shaking water bath. After mixing with lysine (20 mg) for 1 hr, each conjugate was chromatographed in two 5-ml aliquots at 4° on a column of Sephadex G25 in distilled water, and the protein fractions frozen at −70° and lyophilized. Conjugates were passed through a sterile filter (0.2 μm) and centrifuged at 105,000 *g* to remove any protein aggregates.

The degree of conjugation of each conjugate was calculated from the specific activity of the lyophilized conjugates and were found to be 0.5:1 (RSA–[^{3}H]$DNP_{0.5}$, 5:1 (RSA–[^{3}H]DNP_5) and 15:1 (RSA–[^{3}H]DNP_{15}). Contamination of the protein with low molecular weight material (e.g. dinitrophenol and DNP-F) was assessed by thin-layer chromatography of the conjugates on silica plates (0.2 mm Silica G60, Merck, Darmstadt, F.R.G.) in propanol:water:acetic acid (70:30:5 v/v) and hexane:ether (1:1 v/v). More than 90% of the radioactivity of all conjugates remained at the origin in both systems and free DNP-F could not be detected. The purity of protein was assessed further by high performance gel permeation chromatography. Samples were eluted from a TSK G3000SW gel filtration column (Toyo

Soda Mfg Co., Japan) with 0.1 M phosphate buffer (pH 7.4) at a flow rate of 1 ml/min and monitored at 280 nm for protein. The rabbit serum albumin conjugates showed a single peak with a retention time of 14 min which corresponded to the retention time of the major peak of HSA and BSA standards, with traces of higher molecular weight proteins (<10%). The BSA conjugate eluted as a major and minor peak (retention times 14 and 12 min, respectively) which corresponded to the peaks of monomer and dimer present in HSA and BSA standards. No alteration in the ratio of monomer to dimer was observed.

Preparation of dinitrophenylated rabbit serum proteins (RSP–DNP). Autologous rabbit serum protein conjugates were prepared essentially as described previously [9]. Blood (10–15 ml) was obtained from the marginal ear vein and the protein concentration of serum from individual rabbits was estimated by the method of Lowry *et al.* [12]. Serum, equivalent to 250 mg protein, was diluted to 20 ml with 0.1 M phosphate buffer (pH 7.4) and [^{3}H]DNP-F in dioxan (2 ml) was added and the mixture stored for 16 hr at 37°. The conjugates were separated from low molecular weight impurities by either dialysis or chromatography over Sephadex G-25, and then lyophilized. Absence of dinitrophenol and dinitrofluorobenzene was verified by thin-layer chromatography as described above for RSA-DNP conjugates. Epitope densities were determined as the ratio of [^{3}H]DNP (measured radiometrically) to albumin equivalents [9] and were found to be 0.5, 2, 15 and 30.

Disposition of BSA–DNP conjugate in the rabbit. Four male New Zealand White rabbits (3–3.5 kg) were administered BSA-[^{3}H]$DNP_{0.5}$ (50 or 250 mg/kg) in 0.15 M NaCl (10 ml/kg) intravenously via the marginal ear vein. Blood samples (5 ml) were collected into heparin at 1, 2, 4, 7, 8, 9, 10, 11, 14, 17, 21 and 28 days after administration. The plasma was immediately removed after centrifugation and an aliquot (200 μl) was assayed for radioactivity by liquid scintillation spectrometry in 4 ml scintillant. We have shown in previous studies that total radioactivity represents undissociated conjugates [9]. The remainder of the plasma was stored at −20°.

Disposition of RSA–DNP conjugates in the rabbit. Rabbits from which albumin had previously been obtained were administered one of the conjugates prepared from their own albumin. Each rabbit received autologous RSA–[^{3}H]DNP conjugate (50 mg/kg) in 0.15 M NaCl (10 ml/kg), intravenously via the marginal ear vein. Blood samples were collected and treated as above.

Disposition of RSP–DNP conjugates in the rabbit. Rabbits from which serum proteins had previously been obtained were administered the conjugates prepared from their own protein. Each rabbit received autologous RSP–DNP conjugate (50 mg/kg) in 0.15 M NaCl (10 ml/kg) intravenously via the marginal ear vein. Blood samples were collected and treated as above. In addition, the rabbits given RSP–DNP_2 received a second dose of the conjugate (4 weeks after the first injection), and blood samples were collected at regular intervals thereafter.

ELISA for detection of rabbit IgG and IgM anti-BSA antibodies. BSA-specific antibodies (IgG and IgM) in the sera of rabbits administered BSA-DNP conjugates were detected by ELISA. Microtitre plates were coated overnight at 4° with 10 μg/ml BSA 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% w/v Tween 20 (PBS–Tween) and shaken dry. All subsequent washes were performed in the same manner, 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 successively incubated with the following, with washing between each step: 100 μl of test serum, initially diluted 1:20 and serially diluted (1:5) down columns in duplicate; 100 μl of goat anti-rabbit IgG (diluted 1:4000), or 100 μl of goat anti-rabbit IgM (diluted 1:4000); 100 μl of peroxidase-labelled rabbit anti-goat IgG (diluted 1:4000); 100 μl of substrate solution containing 400 μg/ml of *o*-phenylenediamine dihydrochloride (OPD) and 0.1% hydrogen peroxide (30% w/v) in 0.1 M citrate–phosphate buffer (pH 5.0). The enzyme–substrate reaction was terminated after 10 min by addition of 50 μl of 25% v/v sulphuric acid to each well. Absorbances were read at 490 nm using a dual wavelength automated plate reader (Dynatech MR600), with the reference wavelength set at 630 nm. Antibody titres were calculated as the reciprocal of the dilution of antiserum giving half-maximal end point absorbance. Anti-BSA activity was defined as antibody titre following coating of plates with BSA, when no activity was detected against OVA.

ELISA for detection of rabbit IgG and IgM anti-DNP antibodies. DNP-specific IgG and IgM antibodies in sera of rabbits administered DNP-F and DNP–protein conjugates were also determined by ELISA. Microtitre plates were coated overnight at 4° with 100 μg/ml OVA–DNP_{15} or OVA in 0.05 M phosphate buffer (pH 7.2). Sera were initially diluted 1:10 and serially diluted (1:3) down columns in duplicate. All subsequent steps and conditions were as described above for detection of anti-BSA antibodies. Anti-DNP activity was defined as antibody titre (see above) following coating of plates with DNP–OVA, when no activity was detected against OVA alone. Positive IgM and IgG anti-DNP activity was recorded when anti-DNP titres were >20.

ELISA for detection of rat IgG and IgM anti-DNP antibodies. DNP-specific IgG antibodies in the sera of rats administered DNP-F were assayed by ELISA by the same method employed for detection of rabbit antibodies, except that rabbit anti-rat IgG (1:1000) was used in place of goat anti-rabbit IgG. Peroxidase-labelled goat anti-rabbit IgG (1:4000) was used in place of peroxidase-labelled rabbit anti-goat IgG.

RESULTS

Immunogenicity of DNP-F in the rat

The effect of chronic administration of various doses of DNP-F on the production of anti-DNP antibodies in the rat is shown in Fig. 1. After the first series of injections the ED_{50} was 2.7 μmol/kg.

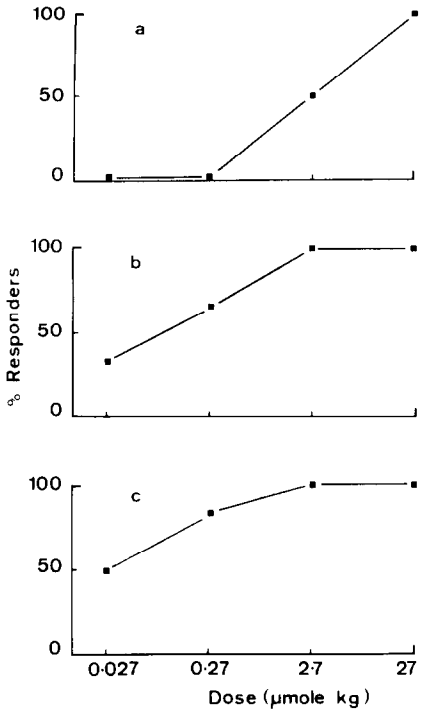

Fig. 1. Immunogenicity of DNP-F in the rat. The data are presented as the percentage responders in each group (N = 4–6 animals) after i.m. administration of various doses of DNP-F for 4 days (a) and after subsequent sets of injections 1 month (b) and 2 months (c) later. IgG anti-DNP antibodies were measured by ELISA 14 days after each set of injections.

Subsequent injections, at monthly intervals, increased the incidence of antibody response. Antibody titres were significantly ($P < 0.05$) greater after the second set of injections, than after the first set of injections. The specificity of the IgG antibodies was confirmed by hapten inhibition with *N*-acetyl-DNP-lysine which reduced binding by >50%.

Disposition and immunogenicity of [^{3}H]*DNP-F in the rabbit*

After intravenous administration of [^{3}H]DNP-F to rabbits, circulating protein conjugates were measured as total radioactivity in serum, since chromatography showed that >95% of [^{3}H]DNP was irreversibly bound to protein. [^{3}H]DNP protein conjugates accumulated during chronic administration of [^{3}H]DNP-F (Fig. 2) and reached steady-state between days 6 and 8. After the final dose of [^{3}H]DNP-F, plasma concentrations declined with a half-life which was similar for the 0.27 μg/kg (7.53 ± 0.07 3 days) and 2.7 μg/kg dose (7.39 ± 0.2 3 days). There was no evidence for dose-dependent kinetics, in terms of either the area under the plasma concentration–time curve or the maximum concentration of conjugate, which was 0.118 ± 0.02% dose/ml for the lower dose and 0.169 ± 0.037% dose/ml for the higher dose. Significant IgG anti-DNP titres (>50) could be measured in both groups of rabbits; the group given 2.7 μmol/kg had higher titres than the group given 0.27 μmol/kg, and the difference was statistically significant from day 19 onwards. IgM anti-DNP antibody titres (range 57–1380) were detected in both groups of animals between days 7 and 25. The specificity of IgG and

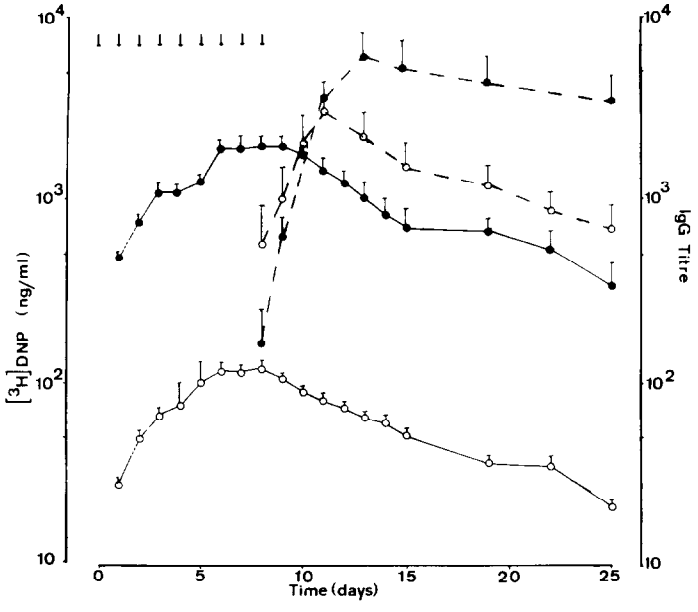

Fig. 2. Plasma disposition and immunogenicity of [^{3}H]DNP-F (0.27 μmol/kg/day ○; 2.7 μmol/kg/day ●) after chronic administration on days 0–8 to male New Zealand White rabbits (N = 4). Plasma concentrations of circulating DNP–protein conjugates (——) were measured as [^{3}H]DNP equivalents. IgG anti-DNP antibody titres (– – –) were determined by ELISA. The results are presented as the mean ± SD.

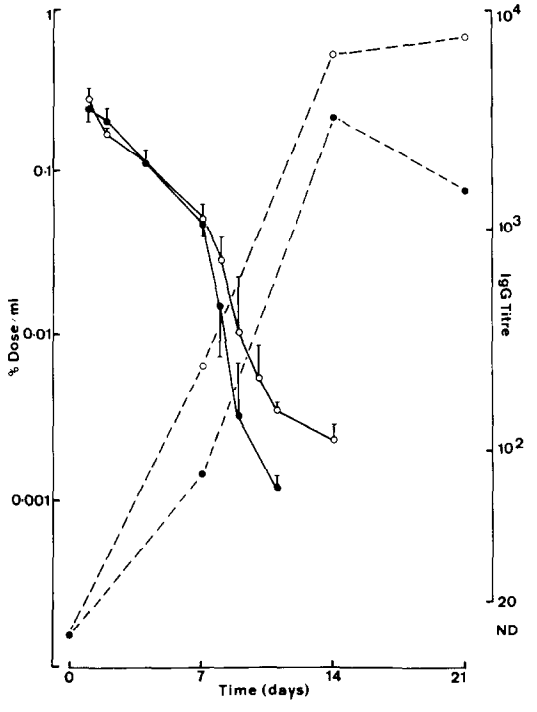

Fig. 3. Plasma disposition and immunogenicity of BSA–[^{3}H]DNP$_{0.5}$ (50 mg/kg ○; 250 mg/kg ●) after a single i.v. injection to rabbits (N = 4). Plasma concentrations of circulating BSA–DNP$_{0.5}$ conjugates are expressed as a percentage of the dose/ml (——) and presented as the mean ± SD. IgG anti-BSA titres (– – –) were determined by ELISA and presented as means, the range of antibody titres is given in the text.

IgM antibodies for DNP was confirmed by hapten inhibition with *N*-acetyl-DNP-lysine (10 μg/ml) which reduced antibody binding by >50%.

Plasma disposition of dinitrophenylated heterologous and autologous protein conjugates in the rabbit

The plasma concentrations of the various conjugates were measured as total radioactivity in plasma, since chromatography showed >95% of [^{3}H]DNP in plasma was irreversibly bound to proteins.

After intravenous administration, plasma concentrations of BSA–DNP$_{0.5}$ (50 mg/kg) underwent a biphasic decline with an initial (days 0–6) half-life of 55 ± 10 hr, followed by a rapid elimination phase between days 8–10 ($t_{\frac{1}{2}}$ ~ 18 ± 6 hr). A similar pharmacokinetic profile was observed with a higher dose (250 mg/kg) of the conjugate (Fig. 3).

The pharmacokinetic profiles for the corresponding DNP conjugates prepared from autologous rabbit albumin, and then injected back into the donor rabbit, are shown in Fig. 4. The plasma concentrations of RSA–DNP$_{0.5}$ and RSA–DNP$_5$ fell in a mono-exponential fashion, with apparent half-lives of 110 ± 12 and 99 ± 16 hr, respectively. Thus there was no evidence for immune clearance of these conjugates. Plasma concentrations of RSA–DNP$_{15}$ fell more rapidly, especially from day 7 onwards. The plasma concentration–time profile is complex and there was no attempt to determine plasma half-lives for RSA–DNP$_{15}$. The plasma AUC for days 1–20 (expressed as % dose/ml plasma × hr ± SD) was significantly less for RSA–DNP$_{15}$ (0.289 ± 0.011), than for either RSA–DNP$_{0.5}$ (1.718 ± 0.147) or RSA–DNP$_5$ (1.234 ± 0.181).

The plasma concentration–time profile for RSP–DNP conjugates is shown in Fig. 5a. The elimination profile for each conjugate appeared to be curvilinear over the period measured and therefore no attempt was made to determine plasma half-lives. However, the plasma clearance is dependent upon epitope density as indicated by the AUC for days 1–14 (expressed as % dose/ml plasma × hr ± SD) which were as follows: RSP–DNP$_{0.5}$ (35.6 ± 9.1), RSP–DNP$_2$ (7.8 ± 6.0), RSP–DNP$_{15}$ (2.3 ± 0.5), RSP–DNP$_{30}$ (0.32 ± 0.05). The AUC for RSP–DNP$_2$ after the second injection was 3.93 ± 2.1.

Immunogenicity of dinitrophenylated heterologous and autologous protein conjugates in the rabbit

The serum antibody titres for IgG and IgM antibodies directed towards BSA following intravenous administration of conjugates to rabbits are shown in Fig. 3. Anti-BSA antibodies were detected in the sera from all rabbits given a single dose (either 50 or 250 mg/kg) of BSA–DNP$_{0.5}$. Both IgM and IgG anti-BSA antibodies were detected from day 7 onwards, but there was no evidence for anti-DNP antibodies (IgG or IgM) in either group. Titre was not dependent on dose over the range used, but showed a wide inter-animal variation. On day 14 the anti-BSA titre

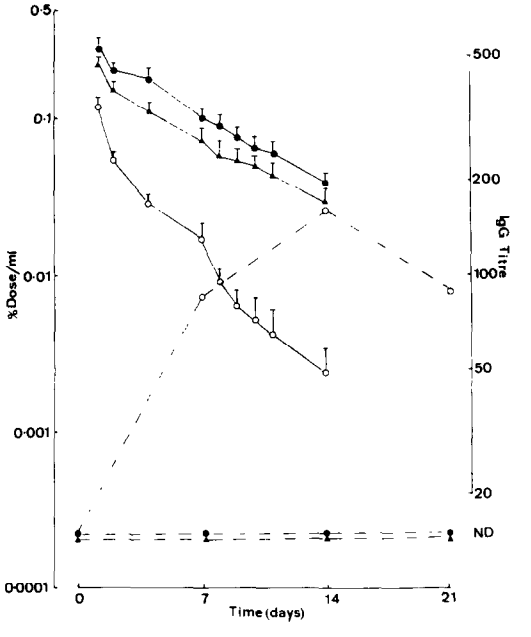

Fig. 4. Plasma disposition and immunogenicity of RSA–[^{3}H]DNP conjugates, after a single i.v. injection to rabbits (N = 4). Plasma concentrations (mean ± SD) are expressed as a percentage of the dose/ml (——) for the conjugates RSA–DNP$_{0.5}$ (●), RSA–DNP$_5$ (▲) and RSA–DNP$_{15}$ (○). The corresponding IgG anti-DNP (– – –) titres were determined by ELISA and are presented as means, the range of antibody titres is given in the text.

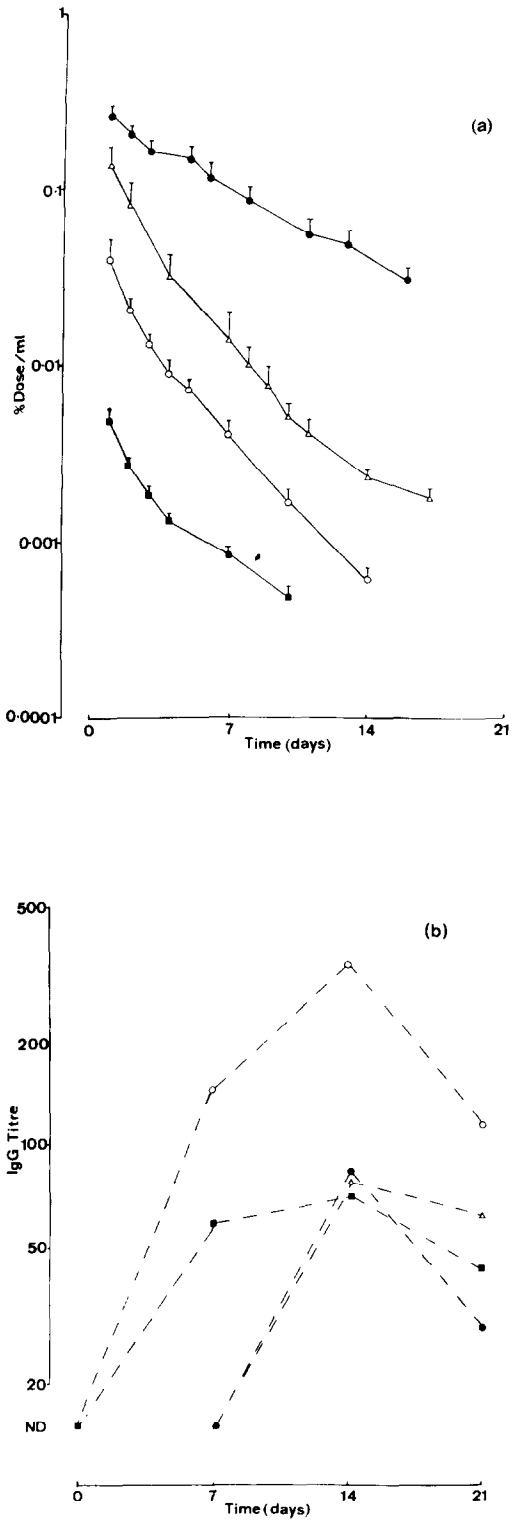

Fig. 5. Plasma disposition and immunogenicity of RSP-[^{3}H]DNP conjugates after a single i.v. injection to rabbits (N = 4). (a) Plasma concentrations (mean ± SD) are expressed as a percentage of the dose/ml (———) for the conjugates RSP–$DNP_{0.5}$ (●), RSP–DNP_2 (△), RSP–DNP_5 (○) and RSP–DNP_{30} (■). (b) The corresponding IgG anti-DNP titres were determined by ELISA and are presented as means, the range of antibody titres is given in the text.

ranges were 1641–4422 (50 mg/kg) and 1552–11,455 (250 mg/kg).

Antibody titres for IgG anti-DNP and IgM anti-DNP (Fig. 3) were measured after administration of conjugates derived from autologous albumin. After administration of the various RSA–DNP conjugates, anti-DNP activity (IgG but not IgM) was detected only in rabbits given RSA–DNP_{15}. On day 14 the titre range was 43–338. The specificity of the antibody response was confirmed by hapten inhibition experiments. The DNP derivative of *N*-acetyl-lysine (10 μg/ml) significantly blocked (>50%) antibody binding.

Anti-DNP antibodies, of both the IgG and IgM classes, were detected in rabbit sera following intravenous administration of all four conjugates prepared from autologous serum proteins. The main IgG antibody titres are shown in Fig. 5b, and the range of IgG anti-DNP titres on day 14 were as follows RSP–$DNP_{0.5}$, 31–199; RSP–DNP_2, 28–135; RSP–DNP_{15}, 46–581; RSP–DNP_{30}, 42–104. The protein:dinitrophenyl ratio (RSP:DNP) was determined as the ratio of protein albumin equivalents:dinitrophenyl residues. Thus it can be seen that over the range RSP–$DNP_{0.5}$ to RSP–DNP_{30} the frequency of IgG anti-DNP antibody response is independent of epitope density and was, in fact, 100%. However, the three highest titres recorded were in rabbits which had received RSP–DNP_{15}. IgM antibodies against DNP were also detected in all four groups, but the frequency of response was variable and the titres very low. Again, the three highest titres recorded were for animals given RSP–DNP_{15}. A more detailed time-course of the response to RSP–DNP_2 after primary and secondary injections is shown in Fig. 6. It can be seen that the inter-animal variation in antibody titre is considerable throughout the study.

DISCUSSION

These studies have shown that administration of a protein-reactive agent, via routes of administration used for drugs, can produce a hapten-specific antibody response of either the IgG or IgM immunoglobulin class. In addition, we have shown that bolus intravenous administration of small quantities of DNP (1–6 μmole/kg) conjugated to autologous serum proteins may also elicit a hapten-specific (DNP) antibody response. It is important to note that in none of these experiments was it necessary to use either an immunological adjuvant or slow-release from a depot injection.

In the rat experimental system (Fig. 1), we studied the relationships between antibody response and the size and frequency of the dose of DNP-F administered. A dose-dependent incidence of IgG antibody response was observed over a 1000-fold dose range, after chronic administration over 4 days. Subsequent treatments increased both the incidence and the titre of the antibody response. Such an experimental scheme should be useful for the assessment of the potential immunogenicity, relative to that of DNP-F, of drugs such as captopril, penicillamine and penicillin which also react directly with proteins [2].

Using the rabbit as an experimental model, it was possible to monitor in detail the time-course of

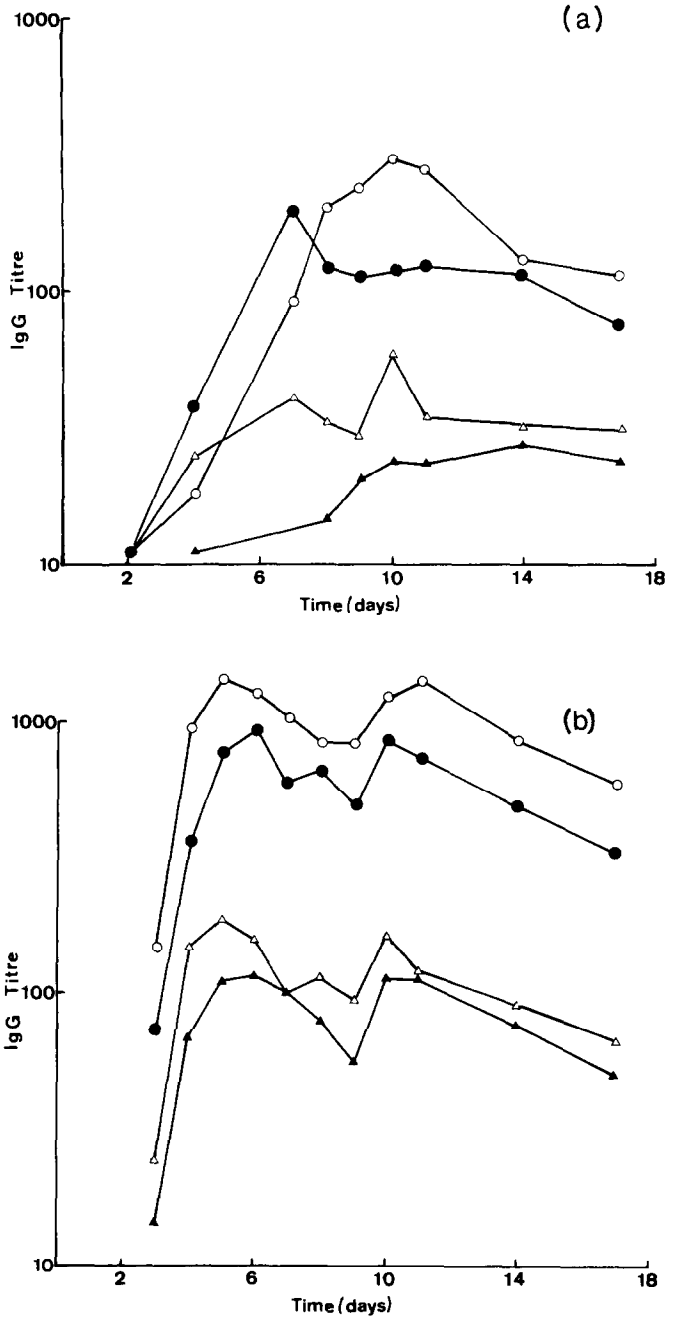

Fig. 6. Time course of IgG anti-DNP antibody response, measured by ELISA, in four rabbits after the first (a) and second (b) injection of RSP–DNP_2.

haptenation of circulating proteins and formation of anti-DNP antibody formation, simultaneously, in the same animal (Fig. 2). Chronic administration of DNP-F for 9 days, produced steady-state plasma concentrations of DNP-protein conjugates. The rate of accumulation and half-life of elimination of [^{3}H]DNP hapten are consistent with the major form of conjugates being derived from albumin. There did not appear to be any dose dependence in the pharmacokinetics of conjugate formation (over the range studied) in keeping with our previous study in the rat [10]. After both doses (0.27 and 2.7 μmol/kg), there was a detectable IgM and IgG antibody titre from day 7 onwards in all animals studied. Higher antibody titres were observed after the 2.7 μmol/kg dose, the difference being statistically significant from day 19.

These experiments show that the presence of a chemical in blood, that can conjugate to proteins, will produce a specific anti-hapten IgG and IgM response. However, the nature of the protein(s) in the conjugate which actually stimulates DNP-specific

lymphocytes (and thus induces antibody production) cannot be elucidated from this type of experiment, since DNP-F becomes covalently bound to extravascular as well as intravascular macromolecules [10].

To study the disposition and immunogenicity of individual haptenated autologous proteins, we adapted a rabbit model for immune complex disease, used by Wilson and Dixon [13] for study of foreign (heterologous) proteins. In their study the foreign protein was labelled with radioactive iodine, whereas for our purposes it was preferable to employ [^{3}H]DNP as the protein labelling group. After administration of BSA–DNP conjugates, a period of slow clearance was followed by rapid clearance between days 8 and 10, which was coincidental with the appearance of IgG and IgM anti-BSA antibodies in serum, which were measured by ELISA (Fig. 3). The rapid elimination is therefore thought to represent immune clearance. We found that essentially similar results were obtained over the dose range 50–250 mg/kg of conjugate.

Having adapted the Wilson–Dixon model for our purposes, we explored the disposition and the immunogenicity of dinitrophenyl conjugated to autologous albumin isolated from, and reintroduced to, the same individual rabbit (Fig. 4).

The plasma clearance of dinitrophenylated autologous albumin conjugates (RSA–DNP) showed a marked dependence upon epitope density. For mean RSA–DNP epitope densities of 0.5 and 5 the half-life of the conjugate was similar to that reported for native albumin in the rabbit [14]; the elimination from plasma was essentially monophasic and there was no evidence for immune clearance at any point. No anti-DNP antibody response was detected after administration of either conjugate. However, increasing the epitope density to 15 altered both the disposition and the immunogenicity of the conjugate. Between days 6 and 8 there was a very marked fall in the plasma concentration of RSA–DNP_{15}, which was coincidental with the appearance of IgM and IgG anti-DNP responses, which were detectable from day 7 onwards. Again, this represents immune clearance of the conjugate since the plasma concentration–time profile is similar to that observed for the clearance of a foreign protein such as BSA, as noted above.

These studies indicate that a considerable degree of conjugation is necessary for recognition of the hapten by the immune system, and also, presumably, for loss of tolerance to autologous albumin [17]. Other workers have shown that DNP conjugated to homologous albumin is also a poor contact sensitizer (T-cell response) in the pig [7]. However, it is important to note that efficient mechanisms exist for the removal and catabolism of chemically altered albumin, which do not involve specific antibody formation [9, 16]. Conjugation to albumin may therefore provide a physiological mechanism for the elimination of electrophilic compounds.

Having investigated the immunogenicity of DNP conjugated to a single isolated autologous protein, we next investigated the effect of conjugation of DNP to total serum proteins which, although more complex, is a closer representation of drug–protein conjugation *in vivo*.

Anti-DNP antibodies were detected in all groups of animals given RSP–DNP conjugates with mean epitope densities from 0.5 to 30 (Fig. 5b). However, it is important to note that the mean ratio (epitope number) presented for RSP conjugates is based on albumin equivalents. Thus proteins with a greater molecular weight than albumin will have correspondingly greater epitope densities, assuming similar chemical reactivity and mean epitope densities may, in fact, be as high as 30. It is also possible that conjugates of individual serum proteins have different intrinsic immunogenicities.

The plasma concentration–time profile for autologous serum proteins (Fig. 5a) showed a marked dependence on the degree of conjugation, as noted in a previous study [9]. It can be seen that increasing the ratio to 2 or greater significantly reduced RSP–conjugate plasma concentrations by day 2; it is thought that this non-immune clearance reflects uptake of chemically altered proteins by Kupffer cells [9], promoted by increased lipophilicity [15]. Thereafter, there was no immune clearance, despite the fact that anti-DNP antibodies could be detected in each group of rabbits. Administration of a second dose of conjugate (for RSP–DNP_2) did not result in a more rapid clearance of conjugate, despite a tenfold increase in antibody titres (Fig. 6).

In conclusion, we have developed a model test system employing DNP-F with which to investigate the potential immunogenicity and disposition of drugs which form conjugates with autologous proteins, avoiding the use of slow release preparations and adjuvants, which are employed by immunochemists to maximize antibody responses. Thus, the system is designed to relate specifically to the formation of drug (metabolite) protein conjugates during therapeutic administration and quantitative assessment of their potential allergenicity. It is anticipated that DNP-F may be employed as a useful positive control, for comparative purposes, in such studies.

Acknowledgements—This work was supported by the Cilag Foundation for Therapeutic Research and Allen & Hanburys plc. B.K.P. is a Wellcome Senior Lecturer. M.D.T. is in receipt of a predoctoral studentship from I.C.I. plc. We are grateful to Dr H. E. Amos for his helpful advice. We would like to thank Mrs C. Clarke for typing the manuscript.

REFERENCES

1. C. W. Parker, *Pharmacol. Rev.* **34**, 84 (1982).
2. H. E. Amos and B. K. Park, in *Immunotoxicology and Immunopharmacology* (Eds J. H. Dean, M. I. Luster, A. E. Musnson and H. E. Amos), pp. 207–228. Raven Press, New York (1985).
3. K. Landsteiner and J. Jacobs, *J. exp. Med.* **61**, 643 (1935).
4. H. N. Eisen, in *Cellular and Humoral Aspects of Hypersensitivity States* (Ed. H. S. Lawrence). Hoeker, New York (1959).
5. B. Rubin, *Eur. J. Immunol.* **2**, 5 (1972).
6. A. Kristofferson, S. Ahlstedt and P. O. Svard, *Int. Archs Allergy appl. Immunol.* **55**, 23 (1977).
7. B. Soeberg, T. Summerska, R. M. Binns and B. M. Balfour, *Int. Archs Allergy appl. Immunol.* **57**, 481 (1978).

8. S. Ahlstedt and A. Kristofferson, *Prog. Allergy* **30**, 67 (1982).
9. N. R. Kitteringham, J. L. Maggs, S. Newby and B. K. Park, *Biochem. Pharmac.* **34**, 1763 (1985).
10. J. L. Maggs, N. R. Kitteringham, P. S. Grabowski and B. K. Park, *Biochem. Pharmac.* **35**, 505 (1986).
11. J. Travis, J. Bowen, O. Tewksbury, D. Johnson and R. Pannel, *Biochem. J.* **157**, 301 (1976).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. C. B. Wilson and F. J. Dixon, *J. Immunol.* **105**, 279 (1970).
14. S. Yedgar, T. E. Carew, R. C. Pittman, W. F. Beltz and D. Steinberg, *Am. J. Physiol.* **244**, E101 (1983).
15. J. B. Lloyd and K. E. Williams, *Biochem. Soc. Trans.* **12**, 527 (1984).
16. C. H. C. M. Buys, A. S. H. de Jong, J. M. W. Bouma and M. Gruber, *Biochem. biophys. Acta* **392**, 35 (1975).
17. S. Sakata and M. Z. Atassi, *Molec. Immunol.* **18**, 961 (1981).