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DRUG–PROTEIN CONJUGATES—XVIII

DETECTION OF ANTIBODIES TOWARDS THE ANTIMALARIAL AMODIAQUINE AND ITS QUINONE IMINE METABOLITE IN MAN AND THE RAT

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Abstract—A specific enzyme-linked immunosorbent assay (ELISA) was developed for the detection and characterisation of antibodies directed against amodiaquine (AQ), an anti-malarial drug associated with agranulocytosis and liver damage in man. The assay incorporated an antigen which was produced by the reaction of amodiaquine quinone imine (AQQI), a protein reactive product produced from AQ by silver oxide oxidation, and metallothionein. The protein-conjugate (AQ–MT) had a ratio of AQ to protein of 5.2:1. Specific anti-drug antibody was defined as the differential binding to AQ–MT and unconjugated MT which was inhibitable by AQ–mercapturate (5 μM). Following administration of AQ (0.27 mmol/kg; for 4 days) to male Wistar rats there was a significant increase in the IgG anti-AQ activity on day 18 ($P < 0.05$, 0.596 ± 0.410, $N = 7$) compared to pre-injection levels (0.111 ± 0.074, $N = 7$). This activity was shown to be specific for the AQ determinant by hapten inhibition with AQ (IC_{50} 250 nM) and AQ-mercapturate (IC_{50} 310 nM). Following administration of AQQI (27 μmol/kg; i.m.; 4 days) there was a significant increase in IgG anti-AQ antibody activities on day 18 (0.584 ± 0.161, $N = 7$) compared to pre-injection levels (0.078 ± 0.048, $N = 7$). This activity was inhibited by AQ (IC_{50} 150 nM) and AQ-mercapturate (IC_{50} 180 nM). In addition IgG anti-AQ antibodies were detected in four patients who exhibited agranulocytosis and one patient who exhibited hepatitis (range 0.017–0.842) whilst receiving AQ at a dose of 400 mg weekly for several weeks, but not in individuals who had not received the drug (-0.014 ± 0.022, $N = 7$). There was no increase in IgG anti-AQ antibody activities in patients who had not exhibited an adverse reaction whilst receiving the drug for the treatment of malaria (-0.059 ± 0.074 on day 0 and -0.053 ± 0.068 on day 7, $N = 13$). Thus, we have shown that AQ is immunogenic in the rat and that the formation of a chemically reactive metabolite (AQQI) is involved in the generation of the antibody response. Furthermore, drug-specific antibodies were detected in sera from five patients with severe adverse reactions to the drug.

Amodiaquine [AQ; Camoquin; 7-chloro-4-(3′-diethylamino-4′-hydroxyanilino)-quinoline] is used for the treatment and prevention of malaria [1], and is particularly effective against some species of chloroquine-resistant *Plasmodium falciparum* [2, 3]. It is thought to act in the same manner as chloroquine, that is either by binding to ferriprotoporphyrin IX and interfering with parasitic degradation of haemoglobin, or by concentration in parasitic lysosomes thus raising their pH and interfering with their activity [4].

AQ is associated with two major side-effects, agranulocytosis and hepatitis [5, 6]. The incidence has been reported to be 1/2000 [5]. The mechanism(s) involved are not clear, but it has been suggested that either inhibition of bone marrow proliferation or immune mechanisms might be involved [7–9].

Previous studies from this department have shown that AQ is readily oxidised to a quinone imine (AQQI), that reacts rapidly and directly with proteins, forming thio-ether conjugates [10]. Therefore, as part of our studies on the hapten hypothesis of drug hypersensitivity, we have developed a specific ELISA, to (1) assess the immunogenicity of AQ and AQQI in experimental animals, and (2) to screen for anti-AQ antibodies in patients who have suffered an adverse reaction whilst taking the drug.

MATERIALS AND METHODS

Chemicals. Amodiaquine (AQ) dihydrochloride dihydrate (Camoquin) was obtained from Parke-Davis (Pontypool, Wales, U.K.). [Quinoline-2-^{14}C]AQ dihydrochloride monohydrate (3.2 mCi/mmol) was synthesised by Amersham International (Amersham, U.K.). Human serum albumin (HSA), keyhole limpet haemocyanin (KLH), equine metallothionein (MT), *o*-phenylenediamine dihydrochloride, *N*-acetyl-DL-homocysteine thiolactone, Freund's Complete and Incomplete Adjuvants were purchased from Sigma Chemical Co. (Poole, U.K.). Polyethylene glycol (PEG), 200 grade was purchased from Fisons (Loughborough, U.K.).

Microtitre plates (Immulon B) were purchased from Dynatech Laboratories (Surrey, U.K.). Rabbit anti-rat IgG (Fc-specific) and horseradish peroxidase-labelled goat anti-rabbit IgG (H and L) were

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† Abbreviations used: AQ, amodiaquine; HSA, human serum albumin; KLH, keyhole limpet haemocyanin; MT, equine metallothionein; HPLC, high performance liquid chromatography; AQQI, amodiaquine quinone imine; FAB, fast atom bombardment; NMR, nuclear magnetic resonance; PEG, polyethylene glycol; ELISA, enzyme-linked immunosorbent assay; Mc, mercapturate.

obtained from Nordic Immunological Laboratories (Maidenhead, U.K.). Goat anti-human IgG (γ-chain) and peroxidase-labelled rabbit anti-goat were obtained from Miles Laboratories (Slough, U.K.). All other reagents were of analytical grade.

The radioactive content of samples was determined in a Packard Tricarb 4640 liquid scintillation spectrometer, using 4 ml scintillation fluid (Aqua-Luma Plus) obtained from May and Baker Chemical Division (Manchester, U.K.).

Analytical methods. Analytical HPLC was performed using an LKB 2150–2152 pump-controller (LKB, Bromma, Sweden) with UV detection at 254 nm (LKB 2151). The column was a 10 μm Bondapak C_{18} column (30 cm × 0.46 cm internal dia.; Waters Assoc., Harrow, Middlesex, U.K.). The eluent employed was a linear gradient of acetonitrile (25–40% over 15 min) in ammonium phosphate buffer (10 mM, pH 4.3) containing heptane sulphonate (5 mM). Fractions (30 sec) were collected and assayed for radioactivity.

Synthesis of amodiaquine derivatives. The quinone imine of AQ (AQQI) was synthesised by a variation of the method of Dahlin and Nelson [11] designed for the preparation of *N*-acetyl-*p*-benzoquinone imine from paracetamol, with modifications as previously described [10]. Silver oxide was prepared by the addition of excess sodium hydroxide (5 M) to an aqueous solution of silver nitrate (5 g in 10 ml distilled water). The resulting precipitate was collected by filtration through paper and washed three times with diethyl ether (10 ml). The solid was then dried over phosphorous pentoxide in a dessicator and stored dessicated until used.

Briefly AQ (0.4 g; 86 mmol) in chloroform (90 ml) over anhydrous sodium sulphate (0.1 g) was stirred with silver oxide (1 g) at room temperature for 30 min. The reaction mixture was then filtered through paper. The filtrate was collected and evaporated to dryness *in vacuo* at 40°. The purity of the material was determined by HPLC.

The three regio-isomeric (2′-, 5′- and 6′-) mercapturates of AQ were prepared from AQQI and *N*-acetylcysteine by a two-phase reaction [12]. The major product was purified to chromatographic homogeneity by preparative reversed-phase HPLC; 80 mg were isolated. The product was fully characterized by UV and i.r. spectroscopy, fast atom bombardment mass spectroscopy (FABms) and high resolution proton nuclear magnetic resonance spectroscopy (NMR).

Synthesis of thiolated human serum albumin. *N*-Acetyl-DL-homocysteine thiolactone (2.1 mmol) was added to HSA (294 μmol) in a 4 ml (0.15 M NaCl/0.01 M phosphate buffer, pH 7.4). When dissolved the pH of the solution was increased by the addition of 2 vol. of 0.1 M glycine in 0.1 M NaCl/0.1 M NaOH. After 2 hr at room temperature, the reaction mixture was chromatographed on a Sephadex G-25 column (30 × 2.5 cm) equilibrated with 0.1 M acetic acid. The flow rate was 1 ml/min, fractions were collected every 5 min. The first seven fractions containing the protein were pooled and then lyophilised. The extent of thiolation was determined using the method of Ellman [13] and the ratio of thiol groups to protein [14] was calculated.

Preparation of amodiaquine-protein conjugates. [^{14}C]AQQI was prepared as described above using [^{14}C]AQ (148 μmol, 0.003 μCi/μmol). The reaction mixture was then centrifuged (2000 *g* for 10 min), the supernatant removed and evaporated to dryness under nitrogen and resuspended in dry dioxan (0.5 ml). Aliquots (5 μl) were analysed by HPLC. The [^{14}C]AQQI (148 μmol) in dioxan (0.5 ml) was then added dropwise to metallothionein (MT; 1.47 μmol) in phosphate buffer (5 ml, 0.05 M, pH 7.4). After reaction for 30 min at room temperature, ascorbic acid (0.1 g) was added to reduce any unreacted AQQI back to AQ. The solution was then dialysed for 2 days against 5 l. distilled water, changed every 24 hr. Duplicate aliquots (1 ml) of the dialysis buffer were taken for liquid scintillation counting in 20 ml of scintillant. The dialysate was then lyophilised.

[^{14}C]AQQI (13 μmol, 0.034 μCi/μmol) was prepared as above in dioxan (0.5 ml). Aliquots (0.25 ml) were added to HSA and thiolated-HSA (147 nmol; ratios of [^{14}C]AQQI to protein of 44 : 1), as described above. Additionally, [^{14}C]AQQI (2.2 μmol, 0.13 μCi/μmol) was added to keyhole limpet haemocyanin (KLH; 20 nmol; ratio of [^{14}C]AQQI to protein of 110 : 1). The reaction mixtures were then dialysed and the dialysates lyophilised as above.

The degree of haptenation was calculated from the amount of radioactivity irreversibly bound to protein, which was determined by the method of Lowry *et al.* [14].

The conjugate produced by the reaction of AQQI with HSA was also analysed by solvent extraction to determine if the dialysis was sufficient for the total removal of unreacted AQ and its products. Aliquots (0.5 ml) of AQ-HSA (1 mg/ml in distilled water) was subjected to exhaustive solvent extraction following precipitation of the protein with ice-cold methanol (4 : 1 v/v) and centrifugation (1000 *g* 10 min). The protein pellets were then washed four times with methanol (2 ml). The final protein pellets were dissolved in NaOH (0.5 M, 1 ml) and aliquots (50–100 μl) were taken for protein determination [14] and for liquid scintillation counting.

Animal immunisation protocols. Male Wistar rats (200–250 g) were treated as follows:—

Group 1: The immunisation protocol for AQ–KLH was similar to that described for paracetamol–KLH by Chesham and Davis [15]. The conjugate (100 μg) emulsified in Freund's Complete Adjuvant (1 ml/kg) was injected i.p. into six male Wistar rats. Fourteen days later the animals received a challenge employing the same dose of conjugate i.p. but emulsified in Freund's Incomplete Adjuvant (1 ml/kg). On days 28 to 31 the animals received four consecutive daily i.p. injection of 100 μg of conjugate in 0.15 M NaCl (1 ml/kg), the animals were killed on day 32. Blood was obtained prior to immunisation (1 ml) and by cardiac puncture on day 32 (5 ml), clotted overnight at 4° and serum separated by centrifugation (1000 *g* 10 min) and stored at −40° until assayed.

Group 2: AQ (0.27 mmol/kg in 1.0 ml/kg PEG) freshly prepared was injected i.m. into seven rats. The injections were performed daily for four consecutive days. Blood samples (1 ml) were obtained

prior to immunisation (day 0) and 2 weeks after completion of the injections (day 18).

Group 3: AQQI (27 μmol/kg in 1 ml/kg PEG), freshly prepared, was injected i.m. into seven rats. The injection schedule and bleeds were as described for rats given AQ.

Human serum samples. Serum was obtained from four patients who had exhibited agranulocytosis, and one who exhibited hepatitis whilst receiving AQ at a dose of 400 mg weekly for several weeks. The serum was obtained during the adverse reaction. The clinical condition of patient 1 was described by Carr [16], patients 2 and 3 had neutropenia whilst receiving AQ as described by Hatton *et al.* [5]. Patient 4 exhibited neutropenia after receiving amodiaquine and proguanil for malarial prophylaxis [17]. Serum was also obtained from a patient who exhibited hepatitis whilst receiving AQ. Serum was also obtained from seven healthy volunteers who had not received the drug.

Serum was obtained from 13 patients from Zambia who had been given AQ for the treatment of malaria. They received AQ at doses of 10 mg/kg on day 0 and then 5 mg/kg, 6, 24 and 48 hr later (total dose of between 1 and 1.5 g). Serum was obtained prior to administration of AQ (day 0) and 7 days later.

Enzyme-linked immunosorbent assay (ELISA) for the detection of rat IgG anti-AQ antibodies. IgG anti-AQ antibody activity in the rat serum samples following administration of AQ–KLH, AQ and AQQI was determined by ELISA. Microtitre plates were coated with AQ–MT or MT (1 μg/ml, optimal coating concentration determined with sera from rats given AQ–KLH, group 1) in 0.05 M phosphate buffer, pH 7.2, overnight at 4° (125 μl/well). The plates were then washed three times with 0.15 M phosphate buffered saline containing 0.05% Tween 20 (PBS–Tween, pH 7.2) and shaken dry. All subsequent washes were performed in the same way. Each well was then successively incubated for 1 hr at room temperature with the following, in a moist box, with washing between each step; 100 μl of pooled rat serum serially diluted down columns in duplicate in PBS–Tween (starting dilution 1/10); 100 μl of rabbit anti-rat IgG (diluted 2000:1 in PBS–Tween); 100 μl of peroxidase-labelled goat anti-rabbit IgG (diluted 5000:1 in PBS–Tween); 100 μl of substrate prepared as previously described [18]. The absorbance was read as previously following termination of the enzyme–substrate reaction after 10 min. IgG anti-AQ activities were calculated as the difference in the optical density (ΔOD) between end-point OD following coating with AQ–MT and unconjugated MT at a serum dilution of 1/30 minus the difference in OD in the absence of serum ($\Delta OD_{1/30} - \Delta OD_0$), as defined previously [19]. ELISA's were also carried out in which microtitre plates were coated with the conjugate produced by the reaction of AQQI and thiolated HSA, and unconjugated HSA (10 μg/ml, 125 μl/well).

Hapten inhibition assays [19] were performed on pooled serum samples obtained from the terminal blood following immunisation with AQ–KLH (day 32) and day 18 following administration of AQ and AQQI. Microtitre plates were coated with AQ–MT (1 μg/ml) as described above. After washing, a range of hapten inhibitors were serially diluted three-fold down columns in duplicate (80 μl/well). To these was added a fixed dilution of serum (20 μl of serum diluted 1:24), to give a final dilution of 1:30, the ELISA was then performed as above. The results were expressed as the IC_{50}, that is the concentration of hapten required to produce 50% reduction in activity.

ELISA for the detection of human IgG and anti-AQ antibodies. The ELISA assays were performed essentially as described for the detection of human anti-benzylpenicilloyl antibodies [19]. Briefly, microtitre plates were coated overnight, with either AQ–MT or MT (1 μg/ml: 125 μl/well). After washing, the wells were incubated with the following, with washing between each step, in a moist box; 100 μl of human serum serially diluted three-fold down columns in duplicate (starting dilution 1/10); 100 μl of goat antihuman IgG (diluted 2000/1); 100 μl of peroxidase-labelled rabbit anti-goat IgG (diluted 5000/1); substrate as previously. The absorbances were read following termination of the enzyme–substrate reaction after 10 min. All serum samples were assayed with AQ–Mc III (5 μM) present at the second step of the assay. The results are expressed as the hapten inhibitable $\Delta OD_{1/30} - \Delta OD_0$.

Statistical analysis. In all cases statistical comparisons between groups were performed using Student's *t*-test for unpaired data. A difference was deemed significant when the P value was less than 0.05. All values quoted in text are mean ± SD, those in figures are mean ± SE.

RESULTS

Characterisation of amodiaquine quinone imine and amodiaquine mercapturate. The product from the silver oxide oxidation of AQ eluted from the HPLC principally as a single homogeneous peak (RT 12 min, previously characterised as AQQI, [10]) a small amount (less than 5%) of unreacted AQ, (RT 9 min) was also detected. Structural characterisation of the major product produced by the reaction of AQQI and *N*-acetyl-cysteine was obtained as described previously [12]. Briefly, positive- and negative-ion FAB spectra contained peaks at *m/z* 517 [M + 1] + and *m/z* 515 [M − 1] −, respectively. The former also contained useful peaks at *m/z* 444 [M − N· $(C_2H_5)_2$]+ and *m/z* 315 [*m/z* 444 − 129]+; loss of *N*-acetylcysteine fragment ($HO_2C \cdot C(CH_2) \cdot NH \cdot COCH_3$). The mercapturates full structure was established by means of 360-MHz proton NMR. Assignment of the point of attachment of the *N*-acetylcysteine moiety, which essentially involved distinguishing between the 5′- and 2′-mercapturates, was achieved using nuclear Overhauser enhancement.

Characterization of amodiaquine protein-conjugates. The epitope densities (drug–protein ratios) of the AQ–protein conjugates synthesized by incubating AQQI and proteins in this study are shown in Table 1. AQQI has been shown to react selectively with sulphydryl groups [10] and thus, AQQI did not readily conjugate to HSA, which only contains one free sulphydryl group [20]. The epitope density of the conjugate following solvent extraction was only

Table 1. The drug–protein ratios (epitope densities) of the protein conjugates produced by incubation of [^{14}C]AQQI and protein

Drug–protein conjugate	Drug–protein ratio
Human serum albumin (HSA)	2.2:1
Thiolated-HSA	13.6:1
Metallothionein (MT)	5.2:1
Keyhole limpet haemocyanin (KLH)	30.0:1

1.6:1. In contrast AQQI did react readily with HSA in which thiol groups had been introduced by chemical modification (ratio of thiol:protein of 73:1), to give a conjugate with an epitope density of 13.6:1. AQQI also conjugated to MT, a protein which contains 20 cysteine residues [21]. The epitope density of the conjugate was 5.2:1, this conjugate was used in the ELISA assays for the detection of anti-AQ antibodies. The greatest ratio (30:1) was obtained by reaction of AQQI with the immunogenic protein carrier KLH.

Characterization of rat IgG anti-AQ antibodies. The KLH–protein conjugate was therefore used to immunise rats, in order to produce a positive antibody response which could be used to develop an ELISA assay for the detection of anti-AQ antibodies.

The IgG antibody response to AQ–KLH given together with adjuvant, was detected by ELISA in pooled serum samples. There was a clear difference in activity between binding to AQ–MT and MT in serum obtained on day 32 (1.124), there was no activity present in serum obtained prior to immunisation (0.066, day 0). There was also differential binding to AQ-(thiolated)-HSA and HSA, the activity on day 32 was 0.608, two-fold lower than that obtained with AQ–MT/MT (1 μg/ml), whilst that on day 0 was 0.024.

The specificity of this activity was confirmed in hapten inhibition experiments as shown in Fig. 1. The IgG activity was inhibited by AQ-(thiolated)-HSA (IC_{50} 5.9 nM), AQ–MT (IC_{50} 175 nM), and AQ–Mc III (IC_{50} 90 nM), but not by unconjugated MT.

There was a significant increase ($P < 0.05$) in the IgG antibody activities following administration of AQ in serum obtained on day 18 ($\Delta OD_{1/30} - \Delta OD_0$; 0.623 ± 0.325, N = 7) compared to day 0 (0.168 ± 0.121, N = 7). This activity was clearly inhibitable by AQ–Mc III at a concentration of 5 μM, which produced at least 90% inhibition of antibody binding to AQ–MT, the hapten inhibitable activity was 0.111 ± 0.074 on day 0 and 0.596 ± 0.410 on day 18.

Detailed hapten inhibition experiments (Fig. 2) revealed that this response was inhibitable by AQ–Mc III (IC_{50} 310 nM) and AQ (IC_{50} 250 nM).

There was a significant increase in the IgG anti-AQ response following administration of AQQI ($P < 0.001$), both the IgG activity and the hapten inhibitable IgG activity on day 18 (1.05 ± 0.524; 0.584 ± 0.161, respectively, N = 7) compared to day 0 (0.156 ± 0.185; 0.078 ± 0.048, respectively, N = 7). The IC_{50} values (Fig. 3) for AQ–Mc III (180 nM) and AQ (150 nM) were of the same magnitude as those obtained with serum obtained following AQ administration.

Human IgG anti-AQ antibodies. The IgG anti-AQ hapten inhibitable (by AQ–Mc III, 5 μM) activities detected in the human samples, along with the clinical data is shown in Table 2. There was a significant difference in the activity of samples from patients who had suffered an adverse reaction whilst receiving AQ compared with samples from individuals who had not received the drug (hapten inhibitable $\Delta OD_{1/30} - \Delta OD_0$: −0.014 ± 0.022; N = 7). Two patients showed a particularly marked response. In addition, there was no increase in the hapten inhibitable specific anti-AQ antibodies in serum from

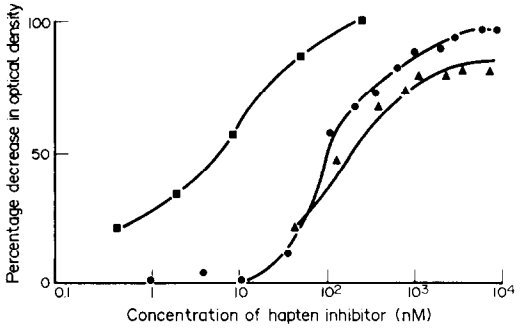

Fig. 1. Hapten inhibition of the IgG anti-AQ activity present in a pooled serum sample (diluted 1/30) obtained on day 32 following immunisation of six rats with AQ–KLH (injection schedule as described in text). The hapten inhibitors were AQ-thiolated-HSA (■), AQ–MT (▲) and AQ–Mc III (●).

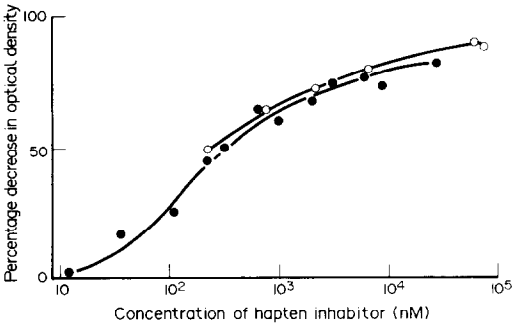

Fig. 2. Hapten inhibition of the IgG anti-AQ antibody activity present in a pooled serum sample (diluted 1/30) obtained on day 18 from seven rats given AQ i.m. (0.27 mmol/kg). Hapten inhibitors were AQ–Mc III (●) and AQ (○).

Table 2. Inhibitable (by AQ-Mc III, 5 μM) IgG anti-AQ activities (inhibitable $\Delta OD_{1/30}$–ΔOD_0) in human serum from patients who had suffered an adverse reaction whilst receiving AQ

Patient	Clinical condition	IgG activity (inhibitable $\Delta OD_{1/30}$–ΔOD_0)
1	Agranulocytosis	0.017
2	Agranulocytosis	0.054
3	Agranulocytosis	0.094
4	Agranulocytosis	0.346
5	Hepatitis	0.842
Controls (no amodiaquine)		−0.014 ± 0.022 (N = 7)
Patients (no adverse reaction)		−0.053 ± 0.068 (N = 13)

patients who had been receiving the drug for the treatment of malaria (−0.059 ± 0.074, N = 13, on day 0 and −0.053 ± 0.068, N = 13, on day 7). Thus, in none of the control samples was there any inhibition of binding to AQ-metallothionein by AQ or AQ–mercapturate.

DISCUSSION

Retrospective epidemiological studies have established that administration of AQ is associated with agranulocytosis and hepatitis. Adverse reactions are more common with prophylaxis compared with treatment so much so, that the drug has now been withdrawn from prophylactic use [5]. However, this may be due to the fact that patients receive a greater accumulative dose of drug during prophylaxis, compared to that received during the treatment of malaria. The mechanism(s) involved in these serious adverse reactions to AQ are not known, and there is no animal model of AQ toxicity presently available. Studies with cells from patients have suggested that the drug may produce dose-dependent inhibition of proliferation of bone marrow cells [8, 9], it has also been suggested that immunological mechanisms may be involved [22].

Previous studies in these laboratories have shown that AQ is readily oxidized to an electrophilic, protein-reactive species amodiaquine quinone imine (AQQI; [10] Fig. 4). This facile oxidation is a consequence of the presence of a *p*-hydroxyaniline moiety adjacent to an aromatic nucleus in the AQ molecule. We have found that AQQI can be generated from AQ by auto-oxidation at neutral pH, peroxidase and silver oxide catalysed oxidation, *N*-chlorination [12] and by stimulated polymorphonuclear neutrophils *in vitro* [23]. This compound has similar chemical properties to *N*-acetyl-*p*-benzoquinone imine, the toxic metabolite formed from paracetamol [24, 25], and also to the model immunogen dinitrofluorobenzene [26]. Thus, formation of AQQI *in vivo* could, in theory, induce adverse reactions either via direct cytotoxicity, or indirectly via initiation of a specific anti-drug immune response, and immune cytolysis of cells bearing specific drug-antigen (Fig. 5).

To investigate the latter mechanism we have devel-

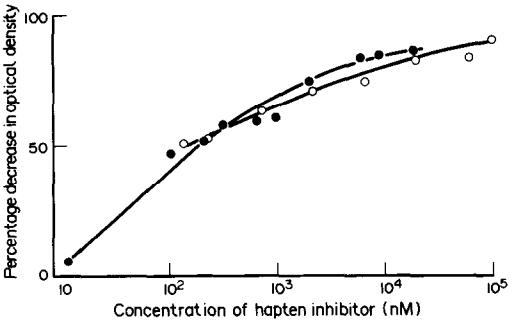

Fig. 3. Hapten inhibition of the IgG anti-AQ activity present in a pooled serum sample (diluted 1/30) obtained on day 18 from seven rats given AQQI i.m. (27 μmol/kg). Hapten inhibitors were AQ–Mc III (●) and AQ (○).

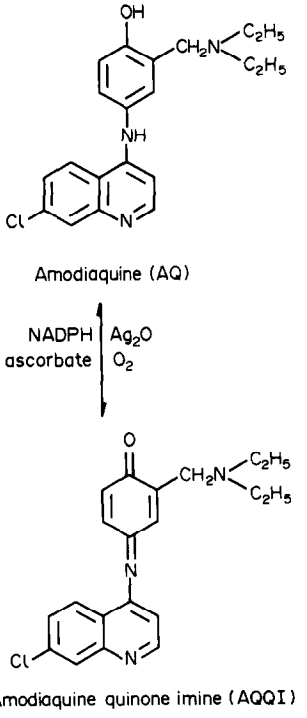

Fig. 4. Structures of AQ and AQQI, and the routes of formation of AQQI from AQ *in vitro*.

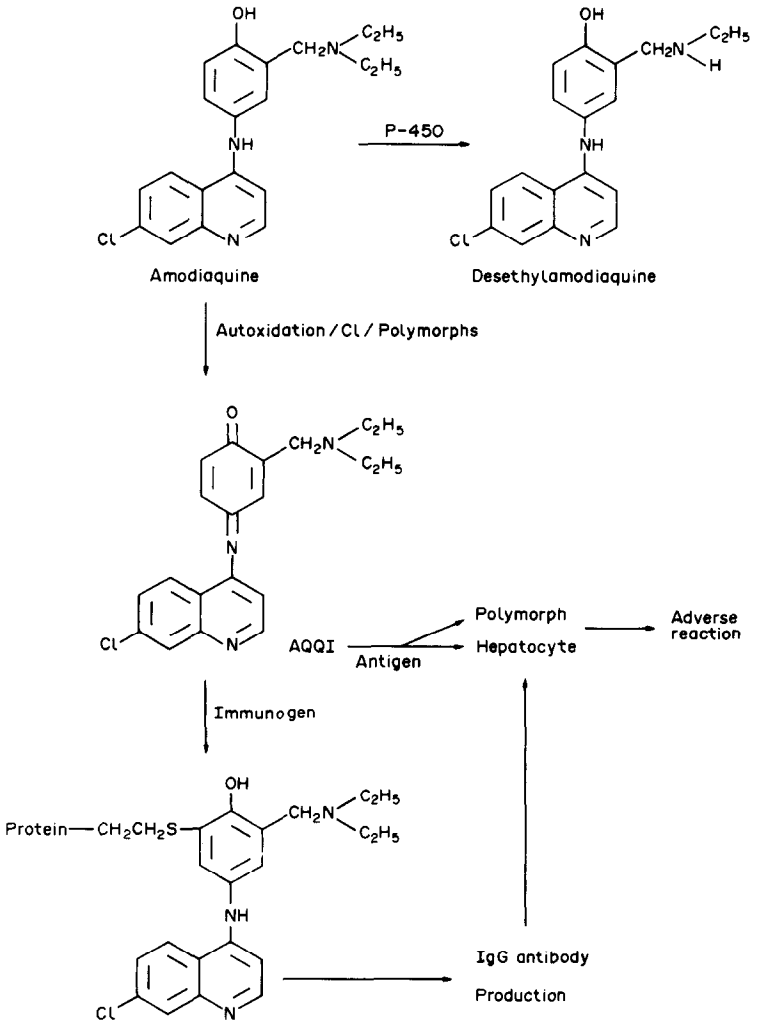

Fig. 5. Scheme summarizing the routes of formation of potential immunogens and antigens formed by the reaction of AQQI and proteins *in vivo* and the specificity of the antibodies produced.

oped an ELISA, in which the test antigen was a protein-conjugate formed from AQQI. Administration of both AQ itself and AQQI produced a specific anti-drug IgG response in the rat. The specificity of the antibody response was confirmed by hapten inhibition experiments with both high molecular and low molecular weight conjugates of AQQI.

A clear specific antibody response was obtained to both AQ and AQQI as indicated by the low IC_{50} values for monovalent hapten-inhibitors (AQ and AQ–McIII), compared to similar values for dinitrofluorobenzene, captopril [27] and penicillin [19]. The most notable feature of the antibody response was the potent inhibition of antibody binding by a synthetic mercapturic acid derivative of AQQI, which was observed for antibodies induced by either AQ or AQQI. This result suggests that quinone imine formation is an obligatory step in the immune response to AQ and serves to emphasise the importance of metabolism in the investigation of drug hypersensitivity. Additionally, the importance of a thorough knowledge of the structure of the hapten for the detection and definition of an antibody response is clearly illustrated.

AQ is extensively metabolized *in vivo* [23] and *in vitro* [10] via a cytochrome P-450 mediated process, to give predominately desethyl–AQ. In contrast to *N*-acetyl-*p*-benzoquinone imine (the reactive species formed from paracetamol), formation of reactive products from AQ does not appear to be mediated by this enzyme system [10]. Nevertheless, protein-reactive AQ derivatives can be generated from AQ by a number of alternative processes. For example, AQ could accumulate in the lysozymes of stimulated neutrophils then be oxidized to AQQI during the release of active oxygen species [23], and reactive derivatives could be formed. Whatever the mechanism of formation we have shown that the formation of AQQI and its subsequent binding gives rise to drug-specific antibodies which recognise thio-ether conjugates of the drug. This scheme is summarised in Fig. 5. In similar studies with paracetamol, we have found that the corresponding quinone imine

(*N*-acetyl-*p*-benzoquinone imine) does not give rise to a detectable antibody response in the rat and have attributed this to the marked instability of the quinone imine derived from paracetamol [29].

The possible clinical relevance of the animal results is shown by the detection of specific IgG anti-AQ antibodies in serum from five patients who exhibited serious adverse reactions after administration of AQ at a dose of 400 mg weekly, for several weeks. Anti-amodiaquine antibodies were not detected in either serum from a control group who had not been exposed to the drug, or a small group of patients who had received AQ for a short period, but did not experience an adverse reaction. A prospective study, with an appropriate control patient group, is now required to define the clinical significance of anti-AQ antibodies.

In conclusion, we have shown that AQ is immunogenic in the rat, and that the formation of AQQI is involved in the antibody of the response. AQ in contrast to penicillin [18] does not react directly with proteins *in vitro*, thus the obligatory role of chemical activation in the immunogenicity of the compound has been demonstrated. Additionally anti-AQ antibodies have been detected in patients who exhibited an adverse reaction whilst receiving AQ, but not in volunteers who had not received the drug. The role of these antibodies in the pathogenesis of adverse reactions can now be determined in a prospective clinical study, using the novel and specific anti-drug antibody assay developed in this study.

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