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The production and characterisation of dinitrocarbanilide antibodies raised using antigen mimics.

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Production and characterisation of polyclonal antibodies to a range of nitroimidazoles

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Abstract

The nitroimidazoles dimetridazole and ronidazole are metabolised to hydroxydimetridazole, while metronidazole is metabolised to hydroxymetronidazole. To screen for a large number of samples by immunoassay for the presence of this family of drugs and metabolites, it was necessary to produce an antibody with broad-spectrum recognition. Metronidazole and hydroxydimetridazole were selected as antigens as they could be coupled to large (immunogenic) carrier proteins at two different positions of the general nitroimidazole structure. The resulting conjugates were used to immunise rabbits, sheep and goats. Seventeen out of thirty-nine animals immunised produced a detectable antibody titre and these antibodies were consequently characterised as regards sensitivity and cross-reactivity.

The panel of antisera produced exhibited IC_{50} ranging from 1.26 to 73.76 ng ml⁻¹ using a competitive ELISA assay. Cross-reactivity studies showed that sera from several animals were capable of significant binding of six of the seven nitroimidazole compounds tested.

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1. Introduction

Nitroimidazoles are used to treat coccidiosis and histomoniasis in poultry. However, they have been suspected of possessing carcinogenic and mutagenic properties and so their use has been restricted within the EU. Under the Council Regulation (EEC) 2377/90 (Appendix IV) of 26 June 1990, the use of dimetridazole (DMZ), ronidazole (RNZ) and metronidazole (MNZ) as veterinary medicines has been prohibited

[1]. More recently under Council Regulation 2205/2001, dimetridazole is no longer permitted as a feed additive [2]. The main metabolites and target residues of these drugs are hydroxydimetridazole (DMZOH) (dimetridazole and ronidazole) and hydroxymetronidazole (MNZOH) (metronidazole) [3].

Methods developed to detect one or more nitroimidazoles have employed gas chromatography–mass spectrometry (GC–MS) [4], liquid chromatography (LC)–MS [5–8] and high performance thin layer chromatography [9]. In order to screen a large number of samples by immunoassay for the presence of this family of drugs, it was necessary to produce an antibody capable of broad-spectrum compound recognition.

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Previously a monoclonal antibody was developed [10] that was capable of binding dimetridazole, hydroxydimetridazole and ipronidazole and in another project two polyclonal antibodies were produced that were capable of binding a range of nitroimidazoles between them [3].

To elicit an immune response in the host animal, small compounds such as the nitroimidazoles must be conjugated to a large (immunogenic) carrier protein. By undertaking a comparative study of the two- and three-dimensional structures of the nitroimidazole group (Fig. 1), hydroxydimetridazole and metronidazole

Fig. 1. Chemical structures of the nitroimidazoles and their metabolites.

were selected as haptens, as they could provide sites for conjugation at two different positions of the general nitroimidazole structure.

The present communication outlines the immune responses elicited in the animals immunised with protein conjugates of these compounds and further characterisation of selected antisera as regards sensitivity and cross-reactivity.

2. Materials and methods

2.1. Reagents and chemicals

Dimetridazole (D4025), ronidazole (R7635), metronidazole (M1547), human serum albumin (HSA) (A1887), bovine thyroglobulin (BTG) (T1001), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (E1769), *N*-hydroxysuccinimide (H7377), 2-(*N*-morpholino)ethanesulphonic acid (M5287), Freund's adjuvant complete (F5881) and Freund's adjuvant incomplete (F5506) were obtained from Sigma (Poole, Dorset, UK). Hydroxydimetridazole, hydroxymetronidazole, ipronidazole and hydroxyipronidazole were provided by the EU Reference Laboratory, BgVV, Berlin, Germany.

Disuccinimidyl carbonate (DSC) (43720) was obtained from Fluka (Gillingham, Dorset, UK), dimethylaminopyrimidine (DMAP) (10770-0) and 2,2'-(ethylenedioxy)bis(ethylamine) (jeffamine) (929-59-9) from Aldrich (Gillingham, Dorset, UK), *p*-maleimidophenyl isocyanate (PMPI) (28100) from Perbio Science (Tattenhall, Cheshire, UK) and horseradish peroxidase (HRP) (814407) from Roche Diagnostics (Lewes, East Sussex, UK). PD-10 gel filtration columns (17-0851-01) were purchased from Pharmacia (Uppsala, Sweden).

2.2. Preparation of immunogens

The first four immunogens were prepared using the cross-linker disuccinimidyl carbonate and incorporating a jeffamine spacer between the drug and the protein.

- (i) Metronidazole–DSC–jeffamine–HSA.
- (ii) Metronidazole–DSC–jeffamine–BTG.
- (iii) Hydroxydimetridazole–DSC–jeffamine–HSA.
- (iv) Hydroxydimetridazole–DSC–jeffamine–BTG.

The carrier protein (20 mg) was dissolved in 2 ml of 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (0.05 M MES, 0.5 M NaCl, pH 4.7) and activated by addition of 5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 3 mg of *N*-hydroxysuccinimide (NHS). Jeffamine (1 M, 100 μ l) was added to the activated protein and allowed to react for 4 h at room temperature. The jeffamine–protein complex was then purified by gel filtration with PBS pH 7.5 using a PD-10 column. The hapten (0.07 mM) was dissolved in acetone and a six-fold molar excess (0.42 mM) of DSC and DMAP, dissolved in acetone, were added to the drug and mixed together for 4 h at room temperature. The acetone was removed by evaporation at room temperature under a stream of nitrogen and the drug derivative re-suspended in 1 ml of PBS, pH 7.5. The drug was now added to the jeffamine–protein complex and allowed to incubate overnight at room temperature. The following day the immunogen was purified by dialysis against 0.15 M saline (3×1 l).

(v) Hydroxydimetridazole–PMPI–jeffamine–BTG.

Hydroxydimetridazole (0.03 mM) was dissolved in 0.5 ml of dimethyl sulphoxide and reacted with an eight-fold molar excess of PMPI in 0.25 ml of dimethyl sulphoxide overnight, at room temperature, under a stream of nitrogen and protected from the light. Water (3 ml) was added to the mixture dropwise to form a precipitate that was suction filtered, washed and freeze-dried. The BTG carrier protein (20 mg) was reacted with jeffamine by the carbodiimide method already described. Again purification was achieved by gel filtration, this time using PBS, pH 7.8. The hydroxydimetridazole–PMPI derivative was dissolved in dimethyl sulphoxide (1 ml) and added to the jeffamine–BTG complex. The mixture was allowed to incubate for 2 h at room temperature before dialysing against 0.15 M saline (3×1 l).

2.3. Preparation of enzyme-labelled drugs

- (a) Metronidazole–DSC–HRP.
- (b) Hydroxydimetridazole–DSC–HRP.
- (c) Hydroxydimetridazole–PMPI–HRP.

The two nitroimidazoles (metronidazole and hydroxydimetridazole) were conjugated to the enzyme label horseradish peroxidase using the same method as for the immunogens (i)–(iv). A third horseradish peroxidase was prepared with hydroxydimetridazole using the same method as for immunogen (v). The jeffamine spacer was excluded in the preparation of all three labels. Purification was achieved by gel filtration using PD-10 columns.

2.4. Preparation of immunogen emulsions

- *Schemes 1 and 3*: The immunogens (1 mg in 2 ml of saline) were added slowly to 2 ml of Freund's adjuvant with vortexing to produce an emulsion. Passing through a narrow bored Pyrex block linking two disposable 5 ml syringes, thickened the emulsions.
- *Scheme 2*: The immunogens (0.2 mg in 0.5 ml of saline) were added to 0.5 ml of Freund's adjuvant and the cocktail mixed vigorously until a homogeneous suspension was obtained.

For all three schemes the first injection of an animal was performed using complete adjuvant, containing heat killed *Mycobacterium tuberculosis*. Subsequent injections were performed using incomplete adjuvant.

2.5. Immunisation of animals

The rabbits used in schemes 1 and 2 were New Zealand whites. Three immunisation schemes were used to produce the antibodies.

- *Scheme 1*: The emulsions were injected subcutaneously into four sites of the animal (left and right front quarters, and left and right hind quarters). Rabbits were immunised every 2 weeks with 1 mg of immunogen, prepared as an emulsion as described and a blood sample was collected 2 weeks after each immunisation from the marginal vein of the ear.
- *Scheme 2*: The second scheme used a smaller amount of immunogen (200 µg). The rabbits were immunised (subcutaneously in four sites as for scheme 1) every 28 days and blood samples were taken 10 days after each immunisation (following the third immunisation) from the marginal vein of the ear.
- *Scheme 3*: Goats were injected subcutaneously and sheep intramuscularly into four sites (left and right

front quarters, and left and right hind quarters). The animals were immunised monthly with 1 mg of immunogen for the first three injections; subsequent injections were given every 3 months with the same amount of immunogen. A blood sample was taken from the jugular vein 1 week after each immunisation.

2.6. Determination of antibody titre

Blood samples taken from the immunised animals were assessed for their antibody content by performing an enzyme-linked immunosorbent assay (ELISA) checkerboard titration using a competitive assay format. Each serum was tested using the three peroxidase conjugates (tracer molecules) produced. The most efficient peroxidase conjugate for each polyclonal, regardless of the immunogen used, was selected. Therefore, the antibodies may be used with a homologous or heterologous enzyme label.

Microtitre plates (Falcon 353070) were coated (100 µl per well) with serial dilutions of untreated serum diluted in assay buffer (1 mM sodium acetate solution, pH 7.2) and incubated overnight at room temperature. The relevant nitroimidazole standard (metronidazole or hydroxydimetridazole) (50 µl) diluted to 1 ng µl⁻¹ in assay buffer and 50 µl of peroxidase conjugate serially diluted in blocking buffer (2 mg ml⁻¹ (w/v) BSA in assay buffer) were added to competitive wells. Control wells containing all components except the competitor were also included. The assay was then incubated at 37 °C for 2 h. After 12 washes with wash buffer (0.9% sodium chloride, 0.1% Tween 20) bound antibody peroxidase conjugate was measured using TMB/E (Chemicon International ES001), a colorimetric substrate. Absorbance was read at 450 nm after 12 min on a Bio-Tek ELISA plate reader. The activity (colour development) in control wells was taken to represent 100%.

2.7. Assessment of antibody sensitivity

The competitive ELISA format (described above) was used to determine the sensitivity of the polyclonal antibodies. The optimum serum and peroxidase conjugate dilutions were predetermined by the checkerboard titration results.

A set of nitroimidazole standards (in the range of 0 to 500 ng ml⁻¹) was prepared in assay buffer and added (50 µl per well). The optimum dilution of peroxidase conjugate was also added (50 µl per well). The average absorbance of zero standard wells (0 STD), containing all components except the competitor, was taken to represent 100% activity and the remaining standards were normalised relative to the absorbance of the zero standard.

2.8. Assessment of antibody specificity

The extent of cross-reactivity with the range of nitroimidazoles was assessed by determining their 50% inhibition of control (IC₅₀) values in the competitive ELISA described above. The cross-reactivity values were calculated as:

cross-reactivity

$$= \frac{\text{IC}_{50} \text{ of nitroimidazole used to raise antibody}}{\text{IC}_{50} \text{ of competing nitroimidazole}} \times 100$$

3. Results

3.1. Antibody titre

While the two metronidazole immunogens were employed in all three immunisation schemes, the initial lack of success of the hydroxydimetridazole immunogens led to the exclusion of scheme 1 for the DSC-coupled immunogens and of schemes 2 and 3 for the PMPI-coupled immunogen. The animals immunised with the metronidazole immunogens and the immunisation schemes used are listed in Table 1. All three of the hydroxydimetridazole immunogens failed to render a detectable immune response. The metronidazole–BTG immunogen produced titres in 83% (10 out of 12) of animals immunised while the metronidazole–HSA immunogen produced titres in 54% (7 out of 13) of animals immunised. It is also interesting to note that all 10 rabbits immunised with low dose metronidazole conjugates (scheme 2 immunisation) produced antibodies compared with only 3 out of 9 with the high dose approach (scheme 1 immunisation).

Sera that exhibited antibody titres were assessed for their sensitivity and specificity. All sera were initially

Table 1

Immunogens and animal numbers used to produce the nitroimidazole polyclonal antibodies

Immunogen	Animal number	Immunisation scheme	Antibody obtained
MNZ–DSC–jeff–BTG	PC100	2	✓
	PC101	2	✓
	PC102	2	✓
	PC103	2	✓
	PC104	2	✓
	R581	1	✓
	R583	1	✓
	R587	1	×
	R589	1	×
	G69	3	✓
	G82	3	✓
	S90	3	✓
MNZ–DSC–jeff–HSA	PC105	2	✓
	PC106	2	✓
	PC107	2	✓
	PC108	2	✓
	PC109	2	✓
	R580	1	✓
	R582	1	×
	R586	1	×
	R588	1	×
	R591	1	×
	S68	3	✓
	G83	3	×
	S84	3	×

Abbreviations: MNZ, metronidazole; DSC, disuccinimidyl carbonate; jeff, jeffamine; BTG, bovine thyroglobulin; HSA, human serum albumin; PC, rabbit from scheme 2; R, rabbit from scheme 1; G, goat from scheme 3; S, sheep from scheme 3; ✓, a viable antibody was produced; ×, a viable antibody was not produced.

tested with each of the three peroxidase conjugates (tracer molecules) produced to determine the most efficient peroxidase conjugate for each polyclonal, regardless of the immunogen used.

The results in Table 2 show that 9 out of the 17 antibodies perform best, with regards sensitivity and cross-reactivity, when used in the heterologous assay format (i.e. different compounds were used to produce the antibody and peroxidase conjugate). All nine sera that showed the best performance in the heterologous format were derived from rabbit. In contrast the highest sensitivities from all goat and sheep sera were found when used in the homologous assay format.

Table 2
Comparison of IC₅₀ values and cross-reactivity profiles of the antibodies

Immunogen	Animal number	Antibody/HRP dilution	IC ₅₀ using HRP stated (ng ml ⁻¹)		Percentage cross-reactivity						
			MNZ–DSC–HRP	DMZOH–PMPI–HRP	MNZ	MNZOH	DMZ	DMZOH	RNZ	IPZ	IPZOH
MNZ–DSC–jeff–BTG	PC100	1:4k/1:16k		1.3	100	811	36	29	24	30	0.2
	PC101	1:4k/1:64k		73.8	100	321	607	635	463	558	63
	PC102	1:4k/1:16k		1.7	100	96	84	22	107	7	<0.01
	PC103	1:4k/1:64k	6.9		100	46	437	73	14	386	4
	PC104	1:4k/1:8k		1.5	100	1579	210	91	32	37	2
	R581	1:8k/1:32k		3.6	100	196	574	240	61	20	4
	R583	1:4k/1:64k		9.6	100	69	626	81	188	148	18
	G69	1:8k/1:20k	1.9		100	4	107	5	0.35	0.02	<0.01
	G82	1:2k/1:40k	2.8		100	48	28	9	98	680	8
	S90	1:4k/1:40k	3.1		100	20	22	10	150	163	3
MNZ–DSC–jeff–HSA	PC105	1:4k/1:64k	21.8		100	45	355	32	15	270	4
	PC106	1:4k/1:16k		6.1	100	47	116	54	31	28	4
	PC107	1:4k/1:64k		6.2	100	14	443	52	75	92	23
	PC108	1:4k/1:4k	12.8		100	17	184	29	6	12	<2
	PC109	1:4k/1:16k		6.9	100	335	145	46	256	260	3
	R580	1:8k/1:16k	7.7		100	6	32	5	4	<0.01	0.01
	S68	1:8k/1:40k	10		100	10	61	19	169	505	9

Abbreviations: MNZ, metronidazole; MNZOH, hydroxymetronidazole; DMZ, dimetridazole; DMZOH, hydroxydimetridazole; RNZ, ronidazole; IPZ, ipronidazole; IPZOH, hydroxyipronidazole; DSC, disuccinimidyl carbonate; jeff, jeffamine; BTG, bovine thyroglobulin; PMPI, *p*-maleimidophenyl isocyanate; HRP, horseradish peroxidase; PC, rabbit from scheme 2; R, rabbit from scheme 1; G, goat from scheme 3; S, sheep from scheme 3.

Fig. 2. Standard curve for metronidazole in 1 mM sodium acetate solution using the rabbit antibody PC102.

3.2. Antibody sensitivity

The 50% inhibition of control values (IC_{50}) for each antibody are shown in Table 2 along with the working dilutions of both the antibodies and their respective enzyme conjugates. PC100 exhibited the highest relative affinity for metronidazole with an IC_{50} of 1.3 ng ml^{-1} ; the three next best were PC104, PC102 and G69 with IC_{50} values of 1.5, 1.7 and 1.9 ng ml^{-1} , respectively. The remaining IC_{50} values ranged from 2.8 ng ml^{-1} (G82) to 73.8 ng ml^{-1} (PC101). A typical standard curve for PC102 is shown in Fig. 2.

3.3. Antibody specificity

The three most sensitive antibodies displayed cross-reactivity profiles that were able to bind a range of nitroimidazoles excluding hydroxyipronidazole. The fourth most sensitive, G69, did not display a

satisfactory profile. Other antibodies such as R581, R583, PC106 and PC109 displayed good cross-reactivity profiles but were not sufficiently sensitive. Table 2 presents the results as percentage cross-reactivity.

4. Discussion

The aim of the project was to produce a generic antibody capable of binding a wide range of the nitroimidazole compounds. The structures of seven nitroimidazoles of interest (Fig. 1) show that conjugation is possible, via the hydroxyl group, at position 1 of metronidazole and position 2 of hydroxydimetridazole and hydroxyipronidazole.

It was decided to produce immunogens using both of these positions. Hydroxydimetridazole was the preferred choice for position 2 conjugation as hydroxyipronidazole is complicated by dimethyl substitution at this position.

The nitroimidazoles are relatively small so a jeffamine spacer was used to extend the distance between the drug and the carrier protein. The extra length of the spacer reduces the effect of steric hindrance on conjugation and makes the hapten a more distinct feature to which the immune system of the host animal can respond.

Seventeen of the 25 animals immunised with a metronidazole preparation yielded an antibody whereas none of the 14 animals treated with hydroxydimetridazole immunogens displayed a response. Clearly conjugation of hydroxydimetridazole to a protein is possible as a viable enzyme label was produced by coupling the drug via PMPI. However, coupling to BTG by this method and to HSA and BTG via the DSC cross-linker failed to produce an immunogen capable of eliciting an immune response. Conjugation of metronidazole occurred at a different position than hydroxydimetridazole and the reactive hydroxyl is displaced further from the ring by an additional $-\text{CH}_2$ group. Consequently, metronidazole may have been presented to the immune system as a more distinct structure from the hapten–protein complex.

The metronidazole–DSC and hydroxydimetridazole–PMPI labels were both viable to different extents with different antibodies. The BTG version of the metronidazole immunogens delivered a higher rate of success than the HSA version and also produced more sensitive antibodies. This result is contrary to previous work performed in this laboratory [11] when only HSA immunogens produced antibodies to nicarbazine (BTG and KLH failed to produce a response).

5. Conclusions

Hydroxydimetridazole was not a suitable hapten to use for the DSC conjugation reaction to prepare either an immunogen or an enzyme label. A more effective approach would appear to be the derivatisation prior to conjugation as described by Stanker et al. [10].

Conjugates produced from metronidazole can produce sensitive antibodies, when conjugated via DSC to a carrier protein, with the capability of binding a range of nitroimidazoles. A specific antibody may be needed to screen for the presence of the metabolite hydroxyipronidazole. It would appear however that the

use of ipronidazole, even prior to the EC ban, was very limited and the main targets of nitroimidazole drug residue screening are included within the panel of analytes with which the antibodies produced have substantial cross-reactivity.

In this study, the carrier protein BTG, when used in the low dose format, resulted in the production of a greater number of suitable antibodies than with the high dose BTG regime or with any of the HSA combinations used. As usual, in a manuscript detailing the successful production of antibodies to a low molecular weight compound, the carrier protein used and the dose of the immunogen administered appear to be of great importance. Unfortunately for the immunochemistry fraternity the relationship between the successful production of antibodies and carrier protein and dose of immunogen used seems to vary widely for no apparent reason.

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