

# Diagnosis of antibody-mediated drug allergy

## Pyrazolinone and pyrazolidinedione cross-reactivity relationships

C. H. SCHNEIDER, M. F. KASPER, A. L. DE WECK, H. ROLLI & B. D. ANGST

University of Bern, Institute for Clinical Immunology, Inselspital, Bern, Switzerland

Based on the 1-phenyl-2,3-dimethyl-3-pyrazolin-5-one series and on the 1,2-diphenyl-pyrazolidine-3,5-dione series of drugs, haptenic reagents and conjugates were synthesized and evaluated by passive cutaneous anaphylaxis in guinea pigs, and by ELISA tests using rabbit antisera against the haptens. No cross-reactivity between pyrazolinone and pyrazolidinedione haptenic reagents was found in any of the test systems. But also within each series, the animal antibodies showed rather strict specificities upon interaction with related haptens or drugs. These results are somewhat unexpected, because the haptens were used in connection with long and flexible spacer arms. Furthermore, they are not typical for certain other drug allergies. The strict specificity reduces the diagnostic potential of the haptenic reagents when used in serological tests or in skin testing.

*Key words:* antigens; antipyrine; diagnosis; phenylbutazone; serology; skin tests.

*Accepted for publication 17 April 1987*

### CLINICAL ASPECTS

The demonstration of antibody involvement in untoward reactions related to pyrazolinone and pyrazolidinedione drugs by means of serological tests or skin testing remains problematic. It may be extrapolated from animal immunizations that the relevant haptenic structures allow only a low order of cross-reactivity, if any at all. Antibodies against derivatives of the original drugs and metabolites may therefore remain undetectable even with highly effective, oligovalent drug-peptide conjugates.

Diagnosis of antibody-mediated allergy involving drugs and other chemicals of low molecular weight is not straightforward, because the relationship between haptenic structure of the drug and that to which antibodies are directed, is in most cases not clear. Elicitation of specific reactions in skin tests requires divalent or oligovalent units which in some cases may form *in situ* by complex formation or chemical conjugation. However, preformed synthetic oligovalent conjugates are expected to be more effective. In serological tests, such as RAST or ELISA, the drugs have likewise to be used in a carrier-attached form.

It seemed conceivable that problems of specificity could be overcome by using haptens

connected to carriers via long and flexible spacer molecules. A hapten displayed in this way should enable interaction of antibody populations with various structural elements of the entire haptenic group and thus allow for considerable cross-reactivity. If this was the case, antibodies even against metabolically modified structures of the original drug might be reliably detected. Our experience with drugs and haptens of the 1-phenyl-2,3-dimethyl-3-pyrazolin-5-one and 1,2-diphenyl-pyrazolidine-3,5-dione series (Figs. 1 and 2) shows however, that "hapten-spacing" is not a general route to enhanced cross-reactivities and seems, in some instances, hardly effective.

The data presented here are an extension of



(9 mmol) N-hydroxysuccinimide plus 9.1 mmol dicyclohexylcarbodiimide in 100 ml  $\text{CH}_2\text{Cl}_2$  was stirred at room temp. for 1 h. Excess carbodiimide was inactivated with acetic acid and the urea derivative was filtered off. The filtrate was evaporated and the residue recrystallized twice from  $\text{CH}_2\text{Cl}_2$ /hexane. Yield: 3.3 g (90%). TLC (chloroform/methanol, 9:1): Rf 0.39, homogeneous; TLC (ethylacetate/acetic acid/ $\text{H}_2\text{O}$ , 3:1:1): Rf 0.77, homogeneous (IN +; UV +). -IR ( $\text{CH}_2\text{Cl}_2$ ): 3050m, 2940w, 1810m, 1780m, 1750s, 1710m, 1590s, 1490s, 1450w.

*N<sup>6</sup> (1,2-Diphenyl-3,5-dioxo-pyrazolidin-4-yl-acetyl)-6-aminohexanoic acid (Butaz)*

To a suspension of 0.82 g (2 mmol) of intermediate 3 in 20 ml tetrahydrofurane, 0.68 g (6 mmol) of 6-aminohexanoic acid in 20 ml 2%  $\text{K}_2\text{CO}_3$  was added with stirring. The pH was adjusted to 9 with 1 M NaOH and after 12 h the tetrahydrofurane was removed *in vacuo*. The residual aqueous solution was diluted with 30 ml  $\text{H}_2\text{O}$ , extracted twice with 50 ml  $\text{CH}_2\text{Cl}_2$ , brought to pH 2 with 6 M HCl and extracted into  $\text{CH}_2\text{Cl}_2$ . The  $\text{CH}_2\text{Cl}_2$ -extract was dried ( $\text{Na}_2\text{SO}_4$ ), evaporated *in vacuo* and the residue was recrystallized twice from  $\text{CH}_2\text{Cl}_2$ /hexane. Yield: 1.0 g (85%), melting point: 138–141°. TLC (toluene/acetic acid, 9:1): Rf 0.16, homogeneous; TLC (ethylacetate/acetic acid/ $\text{H}_2\text{O}$ , 3:1:1): Rf 0.81, homogeneous (IN +; UV +). -IR ( $\text{CH}_2\text{Cl}_2$ ): 3440m, 3400–2400m, 3050m, 2940s, 2860w, 1750w, 1710m, 1690m, 1670s, 1590s, 1530m, 1490s, 1450w. -UV (0.02 M borate buffer, pH 8.9): 258 nm, molar absorption 20000.

Elementary analysis:  $\text{C}_{23}\text{H}_{25}\text{N}_3\text{O}_5$  (423.5) Calculated: C, 65.24; H, 5.95; N, 9.92%. Found: C, 65.20; H, 5.98; N, 9.91%.

*Preparation of N-hydroxysuccinimideester of Butaz (Butaz-OSu)*

Butaz (0.85 g, 2 mmol) plus 2 mmol N-hydroxysuccinimide plus 2.05 mmol dicyclohexylcarbodiimide in 20 ml  $\text{CH}_2\text{Cl}_2$  were stirred for 1 h. The reaction mixture was treated as

described for intermediate 3. Crystallization from  $\text{CH}_2\text{Cl}_2$ /hexane gave 0.6 g (58%) of activated ester. TLC (ethylacetate/acetic acid/ $\text{H}_2\text{O}$ , 3:1:1): Rf 0.93, homogeneous; TLC (chloroform/methanol): Rf 0.35, broad zone (RH +; UV +).

*Preparation of N<sup>4</sup> (6-aminohexanoyl)-4-amidoantipyrine (Phenaz- $\text{NH}_3^+\text{Cl}^-$ )*

4-Aminoantipyrine (1.12 g, 5.5 mmol) and 5 mmol N<sup>6</sup>-tert. butyloxycarbonyl-6-aminohexanoic acid in 20 ml dimethylformamide were stirred at room temp. for 16 h with 5.5 mmol dicyclohexylcarbodiimide and 5 mmol 1,2,3-benzotriazol-1-ol (6). The pH was kept at 10 with triethylamine. After removal of the dicyclohexylurea by filtration, the filtrate was diluted with 100 ml  $\text{CH}_2\text{Cl}_2$  and extracted with 200 ml each of 0.1 M HCl, 2%  $\text{K}_2\text{CO}_3$  and  $\text{H}_2\text{O}$ . After drying ( $\text{Na}_2\text{SO}_4$ ) and removal of the organic solvent *in vacuo*, the residue was kept in 15 ml 90% trifluoroacetic acid at 0° for 30 min in order to remove the tert. butyloxycarbonyl protecting group. Trifluoroacetic acid was removed *in vacuo* and the residue was taken up in 100 ml 0.1 M HCl and extracted with 200 ml  $\text{CH}_2\text{Cl}_2$ . It was then extracted into 500 ml  $\text{CH}_2\text{Cl}_2$  after adjusting the pH to 11 with 5 M NaOH. After drying ( $\text{Na}_2\text{SO}_4$ ) and concentration *in vacuo*, it was chromatographed on a preparative 20 × 20 cm silica gel 60, F 254 plate (Merck) with  $\text{CHCl}_3/\text{CH}_3\text{OH}/17\% \text{NH}_3$  (5:2:2). The main zone was eluted with the same solvent and the product was lyophilized after solvent removal *in vacuo* and dissolution in an equimolar amount of 1 M HCl to give 1.0 g (63%). TLC (ethylacetate/acetic acid/ $\text{H}_2\text{O}$ , 3:1:1): Rf 0.31, homogeneous; TLC (butanol/acetic acid/ $\text{H}_2\text{O}$ /triethylamine, 10:9:10:1): Rf 0.60, homogeneous; TLC ( $\text{CHCl}_3/\text{CH}_3\text{OH}/17\% \text{NH}_3$ , 5:2:2): Rf 0.71, homogeneous (UV +; NH +; IN blue;  $\text{KMnO}_4$  decoloured (C = C)). -IR ( $\text{CH}_2\text{Cl}_2$ ): 3400w, 3200m, 3150m, 2930s, 2860m, 1690–1610s, 1590s, 1490s. -UV ( $\text{H}_2\text{O}$ ): 260nm, molar absorption 9400.

PAL, a bis-penta-L-lysine peptide carrier (7) as

well as Phenaz<sub>6</sub>-PAL and Buto<sub>6</sub>-PAL (4, 5) have been previously described.

#### *Butaz<sub>6</sub>-PAL (Fig. 2)*

To 200 mg PAL in 4 ml 1 % K<sub>2</sub>CO<sub>3</sub>, a suspension of 365 mg Butaz-OSu in 4 ml tetrahydrofuran was added with stirring. The pH was kept at 10 with 1 M NaOH and after 1 h another 182 mg of Butaz-OSu was added as a solid. After 4 h, tetrahydrofuran was removed *in vacuo* and the residual solution was neutralized with HCl and filtered through cotton wool. The filtrate was passed through a 2.5 × 80 cm Sephadex-G-50 (Pharmacia, Uppsala) column, operated with PBS, and subsequently through a similar column of Sephadex-G-25, coarse, operated with H<sub>2</sub>O. Conductivity measurements showed that the conjugate was well separated from inorganic salt. The conjugate fractions were lyophilized: 335 mg (75 %). TLC (butanol/H<sub>2</sub>O/acetic acid/triethylamine, 10:10:9:1): R<sub>f</sub> 0.82, homogeneous (RH + ; NH -). High voltage electrophoresis (5) (0.02 M borate buffer, pH 8.9, 20 min.): 59 mm, anodic movement, homogeneous. Amino acid analysis: Lys<sub>10</sub>: (6-aminohexanoic acid)<sub>6</sub>; found: 10: 5.99.

*Anti-hapten-HSA antisera* from out-bred guinea pigs and rabbits has been described previously (4).

*Passive cutaneous anaphylaxis (PCA)*. The technique of Watanabe & Ovary (9) was used as described (4, 5) for eliciting reactions in anti-hapten-HSA sensitized guinea pigs.

*ELISA technique* has been described before using Buto-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup> and Butaz-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup> for coating the microtiter plates (4). For inhibition studies the anti-Phenaz-HSA antiserum was used at a 1:6000 dilution.

## RESULTS

#### *Synthesis of Butaz and Butaz<sub>6</sub>-PAL*

In order to have available a 1,2 diphenyl-

pyrazolidine-3,5-dione hapten attached to a spacer molecule, the unsubstituted position 4 (Fig. 2) of the pyrazolidinedione ring offered itself as a point for attachment. The starting compound 1,2-diphenyl-3,5-dioxypyrazolidine can be readily prepared (3), but its substitution with short ω-bromo fatty acids up to the hexanoic acid derivative failed when conditions which proved successful for the introduction of bromobutane into the 4-position (2) were used. The procedure with iodoacetic acid described here gives in addition to the desired compound a second reaction product in considerable proportion which we believe to be a disubstituted derivative. The chosen conditions are such that virtually no disubstituted product forms, and the purification problem is therefore essentially reduced to separate unsubstituted starting compound. A relatively low yield has, however, to be accepted. Intermediate 2, carrying the acetic acid substituent, was then further reacted with 6-aminohexanoic acid to Butaz, which represents the desired pyrazolidinedione hapten with a long and flexible spacer arm ready for further chemical couplings. For preparing Butaz<sub>6</sub>-PAL, the carboxyl group of Butaz was converted into the N-hydroxysuccinimide active ester and reacted with PAL according to established methods of peptide chemistry.

#### *Elicitation of passive cutaneous anaphylaxis*

It was shown previously (4) that Butaz-PAL produces PCA reactions after intravenous application which exceed 500 mm<sup>2</sup> blue area in sites which were intradermally injected with 1:1000 dilutions of anti-Butaz-HSA antiserum from either guinea pigs or rabbits. This is likewise true for elicitation of homologous PCA reactions with Buto-PAL and Phenaz-PAL. Table 1 does not therefore list homologous data and is restricted to showing cross-reactivity in PCA sites injected with 1:10 dilutions of antisera. It is evident that neither Butaz-PAL nor Buto-PAL elicit anti-Phenaz reactions. It is particularly remarkable that the rather flexible open-chain structure represented by Buto cannot be accommodated by anti-Phenaz paratopes. Furthermore, Phenaz-PAL does not elicit

anti-Buto reactions. Cross-reactivity is seen with Butaz-PAL which elicits anti-Buto responses. As shown before (4) Buto-PAL also evokes anti-Butaz responses. However, the extent of these cross-reactivities is not very marked.

#### ELISA tests

Microtiter plates coated with Phenaz-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup>, Buto-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup> or Butaz-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup> by means of glutardialdehyde under defined conditions were reacted with anti-hapten antisera from the rabbit in graded dilutions in order to detect specific binding as described before (4). Fig. 3 shows that Phenaz-coated plates bind only antibody to the homologous hapten, whereas Butaz-coated plates bind in addition to the homologous anti-Butaz antiserum also to anti-Buto antibody. The Butaz/anti-Buto specific interaction is confirmed by the PCA data of Table 1. The Buto-coated plates appear to bind in addition to the homologous anti-hapten antiserum also anti-Butaz antibody to a small extent. This binding would be expected in view of earlier PCA data which showed that Buto-PAL could evoke anti-Butaz reactions.

Quantitative data are obtained by assessing the molar concentration of hapten required to inhibit antibody binding to ELISA plates under specified conditions. This is shown in Table 2

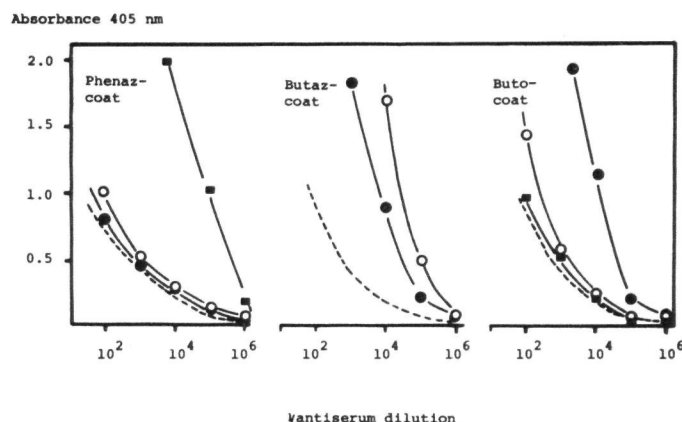


Fig. 3. Binding of anti-hapten-HSA antisera from the rabbit to ELISA microtiter plates coated with Phenaz-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup>, Butaz-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup>, or Buto-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup> as a function of the antiserum dilution (final) in the wells. ■—■, anti-Phenaz; ○—○, anti-Butaz; ●—●, anti-Buto binding; ---- binding of control serum from unimmunized rabbits. The experiments were made twice, giving virtually the same set of data.

for anti-Phenaz antibody, binding to Phenaz-coated plates. Similar data regarding anti-Butaz/Butaz and anti-Buto/Buto bindings have been reported previously (4). It is remarkable that none of the compounds, unless closely related to the Phenaz haptenic structure, shows any measurable inhibition. This includes the salicyl haptens as well as structures of the 1,2-diphenyl-pyrazolidine-3,5-dione type (phenyl-butazone, suxibuzone, Butaz-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup>, Buto-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup>). From the more closely related deriv-

Table 1

Elicitation of PCA in intradermally sensitized guinea pigs\*

| Conjugate<br>i.v.<br>0.1 μmol | Specificity<br>of antiserum | Average area of blueing<br>(mm <sup>2</sup> ) in sites sensitized by<br>1:10 dilutions of |                     |
|-------------------------------|-----------------------------|-------------------------------------------------------------------------------------------|---------------------|
|                               |                             | guinea pig<br>antiserum                                                                   | rabbit<br>antiserum |
| Butaz <sub>6</sub> -PAL       | anti-Phenaz                 | 0                                                                                         | 0                   |
| Buto <sub>6</sub> -PAL        | anti-Phenaz                 | 0                                                                                         | 0                   |
| Phenaz <sub>6</sub> -PAL      | anti-Buto                   | 0                                                                                         | 0                   |
| Butaz <sub>6</sub> -PAL       | anti-Buto                   | 290-340                                                                                   | 60-81**             |

\* For each conjugate 4 animals were used and 3 to 4 sites were injected with the same diluted antiserum. In all animals, one test site was injected with normal rabbit serum 1:10 or normal guinea pig serum 1:10. This was negative in all instances. \*\* Data (4) included for reference.

Table 2

Haptenic inhibition of anti-Phenaz-HSA antiserum, binding to ELISA plates coated with Phenaz-NH<sub>3</sub><sup>+</sup>Cl<sup>-</sup>

| Inhibiting hapten                                   | Concentration (M)<br>for 50 % inhibition |
|-----------------------------------------------------|------------------------------------------|
| Phenaz-NH <sub>3</sub> <sup>+</sup> Cl <sup>-</sup> | 5 × 10 <sup>-6</sup>                     |
| 4-Aminoantipyrine                                   | 1 × 10 <sup>-4</sup>                     |
| Antipyrine                                          | 2 × 10 <sup>-4</sup>                     |
| Metamizole                                          | 1 × 10 <sup>-5</sup>                     |
| Propyphenazone                                      | 1 × 10 <sup>-3</sup>                     |
| Phenylbutazone                                      | > 10 <sup>-2</sup>                       |
| Suxibuzone                                          | > 10 <sup>-2</sup>                       |
| Butz-NH <sub>3</sub> <sup>+</sup> Cl <sup>-</sup>   | > 10 <sup>-2</sup>                       |
| Buto-NH <sub>3</sub> <sup>+</sup> Cl <sup>-</sup>   | > 10 <sup>-2</sup>                       |
| Salicylic acid                                      | > 10 <sup>-2</sup>                       |
| O-Methylsalicylic acid                              | > 10 <sup>-2</sup>                       |
| Tartrazine                                          | > 10 <sup>-2</sup>                       |

atives, propyphenazone is a remarkably weak inhibitor. Tartrazine was included because it contains a sulfonated phenyl residue attached to a di-nitrogen bridge. This constitutes an attachment similar to the one found for the 1-phenyl residue in the Phenaz-related haptens.

## DISCUSSION

Among the antibodies that recognise small haptenic groups there seem to occur at least two types which differ in their way of interaction with these specific structures. An extreme example of one category is constituted by the anti-alcuronium antibodies of Baldo (1) which reacted with a variety of other muscle relaxants and even with unrelated haptens, provided they contained quaternary or tertiary ammonium salt groups. This low-specificity response was found in several cases, but in at least one patient a response strictly directed against alcuronium was also noted. The benzyl-penicilloyl haptenic group also gives rise to an antibody response of quite broad specificity, because the thiazolidine ring as well as the side-chain can be modified without considerable loss of interaction (cf. 5).

Another type of antibody response with rather strict specificity seems involved with the pyrazolinone and pyrazolidinedione antigens studied here. Although the heterocyclic rings in 1,2-diphenyl-pyrazolidine-3,5-dione and in 1-phenyl-2,3-dimethyl-3-pyrazolin-5-one haptenic structures do not differ markedly and, although partial structures around the 1-phenyl substituent are identical, no significant cross-reactivity was observed between the two haptenic series. This was established by the ELISA technique using direct binding as well as haptenic inhibition tests, and confirms data from PCA experiments involving antisera from rabbits and guinea pigs. The only interesting, but weak cross-reactivity, is seen with the Buto haptenic structure which interacts with anti-Butaz antibody and with the Butaz structure which interacts with anti-Buto antibody. On the other hand, the flexible Buto with the opened heterocyclic ring does not interact with anti-Phenaz antibody.

It is remarkable that in the antipyrine series

of drugs, even closely related compounds inhibit rather sluggishly with propyphenazone, requiring as much as  $10^{-3}$  M (Table 2). Weak interaction of propyphenazone with anti-antipyrine antiserum was also reported by others (8) but it is possible that unspecific binding of the drug may compete with antibody binding in the tests.

It may not be expected that the strict specificity of the anti-pyrazolinone and anti-pyrazolidinedione antibodies will hold in all possible cases. However, since we find strict specificities in rabbits, as well as guinea pig immunizations confirming other results in rabbits (8), one should expect that at least a significant proportion of clinical responses will also be characterized by strict specificity. This seems indeed suggested by clinical data obtained from the EGDA (European Group for Drug Allergy). Preliminary surveys show that within a group of 170 patients with a history of adverse reactions to pyrazolinone and pyrazolidine-dione drugs, only 24 % showed positive skin tests to the PAL reagent and 18 % to the bulk components of the incriminated drugs. Of the reactants to the drugs, only a third reacted to PAL conjugates. A more detailed survey of these data will be presented in due course.

## REFERENCES

1. Baldo, B. A. & Fisher, M. M.: Substituted ammonium ions as allergenic determinants in drug allergy. *Nature* 306, 262-264, 1983.
2. Geigy, J. R.: Verfahren zur Herstellung eines Derivates des 3,5-Dioxo-pyrazolidins. Swiss Patent No. 266236, 1950.
3. Geigy, J. R.: Verfahren zur Herstellung des 1,2-Diphenyl-3,5-dioxo-4-n-butyl-pyrazolidins. Swiss Patent No. 308145, 1955.
4. Kasper, M. F., Schneider, C. H., Rolli, H., Angst, D. B. & de Weck, A. L.: Diagnostic reagents in drug allergy: Immunochemical specificity in the 1,2-diphenyl-pyrazolidinedione series. *Immunobiology* 173, 98-109, 1986.
5. Kasper, M. F. & Schneider, C. H.: Antibody-dependent skin reactions towards pyrazolinone and pyrazolidinedione derivatives in the guinea pig. *J. Immunol. Methods* (in press).
6. König, W. & Geiger, R.: Eine neue Methode zur Synthese von Peptiden: Aktivierung der Carboxylgruppe mit Dicyclohexylcarbodiimid unter Zusatz von 1-Hydroxybenzotriazolen. *Ber. Dtsch. Chem. Ges.* 103, 788-798, 1970.

7. Rolli, H. & Schneider, C. H.: Peptide conjugates as diagnostic reagents in clinical immunology. In Theodoropoulos, D. (ed.): Peptides 1987, Proc. 19. Europ. Peptide Sympos. pp. 543-547. de Gruyter, Berlin, 1987.
8. Takatori, T. & Yamaoka, A.: Further production and characterization of antibodies reactive with pyrazolone derivatives. J. Immunol. Methods 35, 147-155, 1980.
9. Watanabe, N. & Ovary, Z.: Antigen and antibody detection by in vivo methods: A reevaluation of passive cutaneous anaphylactic reactions. J. Immunol. Methods 14, 381-390, 1977.

Address:

*Prof. C. H. Schneider*

Institute for Clinical Immunology

Inselspital CH-3010 Bern

Switzerland

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.