ANTIGENICITY TESTS ON PROPIVERINE HYDROCHLORIDE IN GUINEA PIGS AND MICE

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Abstract—Antigenicity of propiverine hydrochloride (P-4), a newly developed drug for pollakisuria, was investigated in guinea pigs and mice.

1. Two strains of mice (BALB/c and C3H/He) showed no production of antibodies against P-4 inoculated with aluminum hydroxide gel (alum) as an adjuvant, judged by the heterologous passive cutaneous anaphylaxis (PCA) test using rats. On the other hand, antibodies against P-4-ovalbumin (OVA) conjugate inoculated with alum was definitely detected.

2. In the studies with guinea pigs, both the inoculation of P-4 alone and of P-4 with Freund’s complete adjuvant (FCA) as an adjuvant did not produce positive reactions in any of homologous passive cutaneous anaphylaxis (PCA), active systemic anaphylaxis (ASA) and passive hemagglutination (PHA) tests. On the other hand, the inoculation of P-4-OVA conjugate with FCA produced positive reaction in all of PCA, ASA and PHA tests.

3. In the active cutaneous anaphylaxis (ACA) test in guinea pigs inoculated with P-4-OVA conjugate with FCA, positive reaction was produced by eliciting injection of P-4-human serum albumin (HSA).

4. In the Schultz-Dale reaction test, any animals in any groups showed no positive reaction to both eliciting antigens, P-4 and P-4-HSA.

5. These findings showed that P-4 had no antigenicity in guinea pigs and mice.

Key words: Antigenicity test, propiverine hydrochloride, guinea pig, mouse.

INTRODUCTION

Propiverine hydrochloride (P-4, 1-methyl-4-piperidyl diphenylpropanoyacetate hydrochloride) is a compound which is developed as a drug for pollakisuria. This

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compound has a spasmylytic effect on the urinary bladder by direct action on the bladder smooth muscle and also by anticholinergic potency (Haruno et al., 1983). In this study, the antigenicity of P-4 was investigated in mice and guinea pigs as a part of its safety research.

MATERIALS AND METHODS

1) Test substances:

P-4 (Lot. No. 011283; M. W., 403.95), synthetized in VEB Sächsisches Serumwerk Dresden, was used as a test compound.

The chemical structure is shown in Fig 1. It is crystalline powder with white or light gray color and moderately soluble in water. P-4 was used by dissolving in distilled water.

![Chemical structure of propiverine hydrochloride (P-4).]

2) P-4-protein conjugate:

It was difficult to make P-4-protein conjugate from P-4. Therefore \(\omega\)-OH-P-4, which has a similar structure of P-4 and has the functional group reacts to the functional group of protein, was used as a starting compound. \(\omega\)-OH-P-4 was coupled to the ovalbumin (OVA) or to human serum albumin (HSA) by making urethane bond through oxycarbonyl chloridation induced with phosgene, to make P-4-protein conjugates (P-4-OVA, P-4-HSA).

Extent of covalent binding of P-4 to the carrier proteins were 3.4 (OVA) and 4.7 (HSA).

3) Adjuvants and Reagents:

Freund's complete adjuvant (FCA, Difco) and aluminum hydroxide gel (16 mg/ml, alum) were used as adjuvants. Alum was prepared in our laboratory.

The following reagents were used: Reserved blood of sheep (Nihon Biotest Laboratory), glutaraldehyde (TAAB Laboratory, 50% solution), Evans Blue (Wako Pure Chemicals), histamine dihydrochloride (Wako Pure Chemicals), sodium azide (NaN₃, Wako Pure Chemicals).

4) Animals:

a) Male Hartley guinea pigs of 3–4 weeks old weighing 247.4–351.0 g were purchased.
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from Hitachi Animal Medical Research Laboratory (Sashima, Ibaraki).
b) Male BALB/c and C3H/He mice of 7 weeks old weighing 21.1–28.8 g and male Wistar rats of 10 weeks old weighing 297.5–353.4 g were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka).

5) Experimental conditions:

The animals were housed in environment controlled rooms at a temperature of 23 ±1°C and at a humidity of 55±10% (for guinea pigs) or 55±5% (for mice and rats). The lighting cycle was 12 hours of alternating light and dark. Commercial feed (guinea pigs: CG-3, mice and rats: CE-2, CLEA Japan Inc., Tokyo) and water were made available for animals ad libitum.

6) Immunization:

a) Guinea pigs

Guinea pigs were injected with 1 mg/kg (group I) and 10 mg/kg (group II) of P-4, which were dissolved in distilled water. In group III (P-4 1 mg/kg), group IV (P-4 10 mg/kg) and group V(P-4-OVA 0.1 mg/kg), solutions of P-4 and P-4-OVA were emulsified with an equal volume of FCA before injection.

Animals in all groups were injected subcutaneously 3 times at weekly intervals.

b) Mice

Mice were injected with 1 mg/kg (group I) and 10 mg/kg (group II) of P-4, and with 0.1 mg/kg of P-4-OVA (group IV). P-4 and P-4-OVA solutions were mixed with an equal volume of alum. In the group III, animals were injected with alum (16 mg/100g B.W.).

Animals in all groups were injected intraperitoneally 3 times at weekly intervals.

7) Antigenicity tests in guinea pigs:

a) Homologous PCA test in guinea pigs

This test was performed according to the method of Ovary (1958). Two weeks after the final sensitization, blood was collected by heart puncture, and sera were obtained. Each 0.1 ml of the guinea pig serum diluted to 10- and 20-fold was injected intradermally into the back of guinea pigs which had been clipped their back hair short. Four hours after the intradermal sensitization, 1 ml of 1 : 1 mixture of P-4-HSA (10 mg/ml) and a 2% solution of Evans blue was injected intravenously. Thirty minutes later, the guinea pigs were bled to death, and were examined for the leakage of dye at the intradermal injection sites. The PCA reaction was judged as positive by a mean diameter of 5 mm or more.

b) Active systemic anaphylaxis (ASA) test

P-4-HSA solution (5 mg/ml) was injected intravenously in a volume of 1 ml to the sensitized guinea pigs in all groups 19 days after the final sensitization. Signs of anaphylaxis were observed for one hour and evaluated by the following criteria.

1. negative : no sign of anaphylaxis
2. mild : licking or rubbing the nose, and ruffling the fur on occasion
3. moderate : weakness, restlessness, sneeze, cough and grunts in addition to the above signs

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4. severe: retching, evacuation, labored respiration, convulsion and prostration in addition to the above signs
5. death
c) Active cutaneous anaphylaxis (ACA) test

Twenty days after the final sensitization, 0.1 ml of P-4 (10 μg/ml), P-4-HSA (10 μg/ml), HSA (10 μg/ml) and distilled water were injected intradermally into the back of sensitized guinea pigs. Skin reaction was examined at 2, 4, 8, 24 and 48 hrs after the intradermal injection, and evaluated with the degree of reddening and incidence of necrosis.

Evaluation was made by the following criteria.
−: Same redness as injection site of distilled water
±: Slightly more reddening compared with distilled water
+: Clear reddening compared with distilled water
#: Severe change accompanied with necrosis
d) Schultz-Dale reaction test

This test was carried out about three weeks after the final sensitization. After a blow on the head of the sensitized guinea pigs to death, the ileum was isolated and set in aerated Tyrode's solution at 37°C. Contraction of the ileum preparation was measured with histamine dihydrochloride (10^{-7} g/ml) was added at 7 minutes interval till the extent of contraction was stabilized. About ten minutes later, P-4 or P-4-HSA were added at a concentration of 10^{-5} g/ml.
e) Passive hemagglutination (PHA) test

Passive hemagglutination test using sheep red blood cells (SRBCs) was performed with the same serum used in homologous PCA test. The SRBC was sensitized with P-4-OVA according to the method of Avrameas et al (1969).

The normal SRBCs were washed three times with 0.15M phosphate buffered saline (PBS, pH7.2).

SRBCs were suspended to make 4% suspension in PBS containing 2 mg/ml of P-4-OVA. Then 1/10 volume of 2.5% glutaraldehyde solution was added to this 4% SRBC suspension, and the mixture was stirred at room temperature for 1 hour. The cells were washed 3 times with PBS and resuspended to make 1% with PBS containing 1% normal guinea pig serum previously treated by heating at 80°C for 1 hour and put in cold water (0°C) and stored at 4°C after an addition of NaN₃ to a concentration of 0.1%.

Hemagglutination titers of the sera were determined by microtiter method. Each 25 μl of test sera was diluted 1–1024 times by doubling dilution method with PBS containing 1% normal guinea pig serum. Then 25 μl of P-4-OVA-coated SRBC was dropped into each well of the microtiter plate. After stirred and sealed, the plates were left to stand overnight at room temperature. The antibody titer was determined from the maximum dilution showing positive reaction.
8) Antigenicity test in mice:
a) Heterologous PCA test in rats
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This test was performed according to the method of Mota and Wong (1969). About two weeks after the final inoculation, blood was collected from the abdominal aorta of the mice (both BALB/c and C3H/He strain) and sera were obtained. Each 0.1 ml of the mouse serum diluted to 10- and 20-fold was injected intradermally into the back of rats. Twenty-four hours later, 1 ml of 1 : 1 mixture of P-4-HSA (10 mg/ml) and a 2% Evans blue solution was injected intravenously. Thirty minutes later, rats were bled to death, and were examined for the leakage of dye at the intradermal injection sites. The PCA reaction was judged as positive by a mean diameter of 5 mm or more.

RESULTS

1) Antigenicity tests in guinea pigs:

a) Homologous PCA test in guinea pigs

The results are shown in Table 1. In group I (P-4 1 mg/kg) and group II (P-4 10 mg/kg) sensitized with P-4 alone, and in group III (P-4 1 mg/kg) and group IV (P-4 10 mg/kg) sensitized with P-4 and FCA as an adjuvant, the PCA reaction was negative by the challenge of P-4-HSA. On the other hand, in group V sensitized with P-4-OVA (0.1 mg/kg) emulsified with FCA, antibodies were detected in 4 of 5 sera.

b) PHA test

The results are shown in Table 1. In groups I to IV, all of sera showed negative reactions. In group V (P-4-OVA 0.1 mg/kg+FCA), all of sera showed positive reactions with 1024-fold or more PHA antibody titers when P-4-OVA coated SRBCs were used.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sensitization</th>
<th>Dose x3times</th>
<th>Route</th>
<th>No. of Animals</th>
<th>PCA titer</th>
<th>PHA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>P-4</td>
<td>1 mg/kg</td>
<td>s.c.</td>
<td>5</td>
<td>&lt;10</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>P-4</td>
<td>10 mg/kg</td>
<td>s.c.</td>
<td>5</td>
<td>&lt;10</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>P-4+FCAa</td>
<td>1 mg/kg</td>
<td>s.c.</td>
<td>5</td>
<td>&lt;10</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>P-4+FCA</td>
<td>10 mg/kg</td>
<td>s.c.</td>
<td>5</td>
<td>&lt;10</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>P-4-OVA+FCA</td>
<td>0.1 mg/kg</td>
<td>s.c.</td>
<td>5</td>
<td>&gt;20(4)c</td>
<td>&gt;1024</td>
</tr>
</tbody>
</table>

a) Seep red blood cells were coated with P-4-OVA.
b) Freund's complete adjuvant.
c) Figures in parenthesis indicate the number of animals. Challenging antigen : P-4-HSA 5mg/body/ml.

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c) ASA test

The results are shown in Table 2. Anaphylactic sign was not observed in groups I to IV by challenging with P-4-HSA. In group V (P-4-OVA 0.1 mg/kg + FCA) challenged with P-4-HSA, all animals showed anaphylactic signs and 4 of 5 animals died within 10 minutes.

d) ACA test

The results are shown in Table 3. No skin reaction was observed in groups I to V by intradermal injection of distilled water. Slight reddening was observed in each one animal of group I, IV, and V respectively by injection of P-4(1 μg/site). Clear reddening was observed only in animals of group V from 4hr to 48 hr by injection of P-4-HSA (1 μg/site). By the injection of HSA(1 μg/site), slight reddening was observed in one animal of group II. In group V, 1 of 3 animals showed clear reddening from 4hr to 48hr and the other 2 animals showed slight reddening from 4hr to 8hr or from 4hr to 48hr, respectively.

e) Schulz-Dale reaction test

The results are shown in Table 4. The ileum isolated from the animals sensitized P-4 and P-4-OVA (group I-V) did not contract after the addition of P-4 or P-4-HSA (final concentration in water bath was 10⁻⁵ g/ml). In all groups, contraction of ileum was observed after addition of histamine dihydrochloride in a concentration of 10⁻⁷ g/ml (final concentration).

2) Antigenicity test in mice:

a) Heterologous PCA test in rats

The results are shown in Table 5. In groups I to III, the PCA reaction was negative in all test sera challenged with P-4-HSA. In group IV (P-4-OVA 0.1 mg/kg + alum), IgE antibodies were detected in all 5 mice challenged with P-4-HSA.

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### Table 2. Active systemic anaphylaxis test in guinea pigs sensitized with P-4 or P-4-OVA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sensitization</th>
<th>Dose x3times</th>
<th>Route</th>
<th>No. of Animals</th>
<th>Grade of symptom</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>P-4</td>
<td>1 mg/kg</td>
<td>s.c.</td>
<td>5</td>
<td>- 0 0 0 0</td>
</tr>
<tr>
<td>II</td>
<td>P-4</td>
<td>10 mg/kg</td>
<td>s.c.</td>
<td>5</td>
<td>5 0 0 0</td>
</tr>
<tr>
<td>III</td>
<td>P-4+FCA</td>
<td>1 mg/kg</td>
<td>s.c.</td>
<td>5</td>
<td>5 0 0 0</td>
</tr>
<tr>
<td>IV</td>
<td>P-4+FCA</td>
<td>10 mg/kg</td>
<td>s.c.</td>
<td>5</td>
<td>5 0 0 0</td>
</tr>
<tr>
<td>V</td>
<td>P-4-OVA+FCA</td>
<td>0.1 mg/kg</td>
<td>s.c.</td>
<td>5</td>
<td>5 0 1 0 4</td>
</tr>
</tbody>
</table>

Challenging antigen: P-4-HSA 5mg/body/ml. a) Freund's complete adjuvant.
- : No symptom or mild ± : Moderate + : Severe ++ : Death

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### Table 3. Active cutaneous anaphylaxis test in guinea pigs sensitized with P-4 or P-4-OVA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sensitization</th>
<th>Animal No.</th>
<th>P-4</th>
<th>P-4-HSA</th>
<th>HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Compounds</td>
<td>Dose x3times</td>
<td>Route</td>
<td>2 a)</td>
<td>4</td>
</tr>
<tr>
<td>I</td>
<td>P-4</td>
<td>1 mg/kg</td>
<td>s.c.</td>
<td></td>
<td>±</td>
</tr>
<tr>
<td>II</td>
<td>P-4</td>
<td>10 mg/kg</td>
<td>s.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>P-4+FCA b)</td>
<td>1 mg/kg</td>
<td>s.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>P-4+FCA</td>
<td>10 mg/kg</td>
<td>s.c.</td>
<td></td>
<td>±</td>
</tr>
<tr>
<td>V</td>
<td>P-4-OVA+FCA</td>
<td>0.1 mg/kg</td>
<td>s.c.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Time after the i.d. challenge injection (hr).
b) Freund's complete adjuvant.

Dose of challenging antigens, P-4, P-4-HSA and HSA: 1μg/site.

- : Same redness as injection site of distilled water.
± : Slightly more reddening compared with distilled water.
+ : Clearly reddening compared with distilled water.
Table 4. Schultz-Dale reaction of the ileum of guinea pigs sensitized with P-4 or P-4-OVA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Compounds</th>
<th>Dose x3times</th>
<th>Route</th>
<th>No. of Animals</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>P-4</td>
<td>1 mg/kg</td>
<td>s.c.</td>
<td>2</td>
<td>P-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P-4-HSA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Histamine</td>
</tr>
<tr>
<td>II</td>
<td>P-4</td>
<td>10 mg/kg</td>
<td>s.c.</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>P-4+FCA</td>
<td>1 mg/kg</td>
<td>s.c.</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>P-4+FCA</td>
<td>10 mg/kg</td>
<td>s.c.</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>P-4-OVA+FCA</td>
<td>0.1 mg/kg</td>
<td>s.c.</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

a) Freund's complete adjuvant.
- : Negative reaction + : Positive reaction

Table 5. Heterologous passive cutaneous anaphylaxis test in rats for the serum of BALB/c and C3H/He mice sensitized with P-4 or P-4-OVA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Compounds</th>
<th>Dose x3times</th>
<th>Route</th>
<th>Challenging antigen</th>
<th>No. of Animals</th>
<th>PCA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>P-4+Alum</td>
<td>1 mg/kg</td>
<td>i.p.</td>
<td>P-4-HSA</td>
<td>5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>II</td>
<td>P-4+Alum</td>
<td>10 mg/kg</td>
<td>i.p.</td>
<td>P-4-HSA</td>
<td>5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>III</td>
<td>Alum</td>
<td>-</td>
<td>i.p.</td>
<td>P-4-HSA</td>
<td>5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>IV</td>
<td>P-4-OVA+Alum</td>
<td>0.1 mg/kg</td>
<td>i.p.</td>
<td>P-4-HSA</td>
<td>5</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

a) Dose of P-4-HSA : 5mg/ml/body.
b) Aluminum hydroxide gel.

DISCUSSION

In this work, antigenicity of P-4 was studied with guinea pigs and mice. IgE antibody production in mice was examined by the method of heterologous PCA using rats, and only the mice sensitized with P-4-OVA showed production of IgE antibodies. Therefore, it is considered that P-4 has no immunogenicity in mice. P-4 also showed no antigenicity in guinea pigs, in the studies of PCA, ASA and PHA tests. In the Schultz-Dale reaction test, the ileum from the animals sensitized with P-4 or P-4-OVA did not show the positive reaction by the challenge of P-4 and P-4-HSA. The reason why the ileum sensitized with P-4-OVA had no positive reaction by the challenge of P-4-HSA is considered that intestinal smooth muscle contraction was prevented by the relaxing effect of P-4.

On the protein binding of P-4 and serum albumin, binding ratio to human serum
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albumin (HSA) was 66 ± 5.3% (concentration of P-4: 5–30 μg/ml), binding constant (K) was 3.2x10^3 l/mol and binding number (N) was 1.3 (Mohr et al., 1976). On the other hand, binding constant of various drugs to HSA was 10^4–10^5 l/mol (Hanson, 1981). Hence, it is difficult to consider that P-4 combines with HSA covalently and behaves as a hapten in vivo. From the results of the present study and above considerations, P-4 may be free of antigenicity in clinical use.

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REFERENCES