DELAYED TYPE HYPERSENSITIVITY FOR PENICILLIN IN MICE

I. INDUCTION AND CHARACTERIZATION OF DELAYED TYPE HYPERSENSITIVITY FOR PENICILLIN IN MICE

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The delayed type hypersensitivity (DTH) for benzylpenicilloyl (BPO) group was observed in the footpad swelling reaction (FSR) in mice. Mice were subcutaneously immunized with 300 μg of BPO-human serum albumin (HSA) conjugate and Freund's complete adjuvant and challenged into footpads with 25 μg of BPO-bovine gamma globulin (BGG) conjugate 2 weeks after the immunization. The strongest FSR was observed 24 hours after the challenge. This FSR was typical DTH. Namely, the kinetics of FSR and the histological study showed the pattern of the DTH. Furthermore, the FSR could be transferred to normal syngenic mice by transfer of antigen-primed spleen cells and could not be transferred by anti-Thy-1,2-serum treated cells. The DTH for BPO was observed on day 4 after the immunization and reached the maximum on day 11 to 14. Thereafter, the DTH for BPO decreased gradually in proportion as the IgG antibodies for BPO were produced. C57BL/6 and C3H/He mice high responders, A/J mice moderate, and BALB/c and DBA/2 mice were low responders. Penicillins were broad cross-reactive in FSR and its desensitization test because the DTH for penicillins contained the common reactivity for the penicilloyl moiety. The DTH for BPO was suppressed by intravenous preadministration of HSA and this suppression was sensitive to cyclophosphamide.

Penicillins and cephalosporins are potentially capable of inducing all types of allergic reactions as defined by COOMBS and GELL1). Type I, II and III allergy are thought to be elicited by humoral antibodies such as IgE, IgG and IgM antibodies. Type IV allergy is elicited by the cell-mediated immunity. The properties of the IgG antibodies for penicillins and cephalosporins have been closely investigated in detail2~6). Moreover, we have studied the properties and antigenic specificities of the IgE antibodies for penicillins and cephalosporins in rats. The difference between the antigenic specificities of IgG and IgE antibodies was described previously.7) Namely, the IgG antibodies for penicillin could react with both the acyl side chain moiety and the penicilloyl moiety of penicillin, but the rat IgE antibodies for penicillin could react only with the acyl side chain moiety. However, the cell-mediated immunity for penicillins and cephalosporins has not been well-studied yet. The present studies are, therefore, designed to investigate the induction and characterization of the delayed type hypersensitivity (DTH) for penicillins in mice whose DTH has been clarified by several reports.8)

Materials and Methods

Animals

Ta: A/J, Ta: BALB/c, Ta: C3H/He, Ta: C57BL/6 and Ta: DBA/2 mice were bred at Takeda Chemical Industries, Ltd., Osaka, Japan. Mice were routinely used at 7~9 weeks of age for the immunization.

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Chemicals
Penicillin G (PCG), ampicillin (ABPO) and sulbenicillin (SBPO) were obtained from commercial sources of Takeda Chemical Industries, Ltd., Osaka, Japan. 6-Formamidopenicillanic acid (FPC) was synthesized in Takeda Chemical Industries, Ltd. Human serum albumin (HSA) and bovine gamma globulin (BGG) were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

Preparation of Penicilloyl-protein Conjugate
One hundred mg of HSA or BGG was dissolved in 10 ml of physiological saline, and penicillin was added at ratio of 50 ~ 2,000 μg vs. 1 μg protein. The reaction mixture was adjusted at pH 10.5 with 1 N NaOH. After 24 hours incubation at 37°C, the reaction mixture was dialysed at 4°C for 3 days continuously against 5 liters of physiological saline and then penicilloyl-protein conjugate was chromatographed on Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (3 × 100 cm). The epitope densities of penicilloyl groups in penicilloyl-protein conjugates were measured by the penamaldate method. Penicilloyl-protein conjugates used in this study were benzylpenicilloyl (BPO) 3, 6, 8, 15, 30-HSA, ampicilloyl (ABPO) 28-HSA, sulbenicilloyl (SBPO) 32-HSA, 6-formamidopenicilloyl (FPO) 19-HSA, BPO 4, 8, 18, 30, 51-BGG, ABPO 50-BGG, SBPO 38-BGG and FPO 30-BGG.

Immunization
Following the method of SNIPPE 10), mice were subcutaneously immunized with 0.2 ml of the emulsion of 3 ~ 3,000 μg of penicilloyl-HSA and Freund’s complete adjuvant (FCA: Difco). Elicitation of the footpad swelling reaction (FSR) and its measurements. Mice were intradermally challenged into the left hind footpad with 0.04 ml of 0.4 ~ 100 μg of penicilloyl-BGG saline solution 4 ~ 30 days after the immunization. The same volume of saline was injected into the right hind footpad. The degree of the FSR was estimated by the method of KERKHAERT 11). The thickness of both footpads was measured 3 to 72 hours after the antigen challenge, using the dial thickness gauge (Peacock). The FSR was represented as the difference between the thickness of the left and right footpad.

Measurement of Serum Antibodies for the Penicilloyl Group
Penicilloyl-BGG sensitized sheep red blood cells (SRBC) were prepared by the glutaraldehyde-tannic acid method 12) and serum antibodies for penicilloyl group were measured by the micro-titer method.

Histological Study
Hind footpads were fixed in 10% neutral formalin 48 hours after the antigen challenge, embedded in paraffin, sectioned at 5 μ thickness and stained with hematoxylin-eosin. Lightmicroscopical observation was performed.

Transfer of the Footpad Swelling Reaction (FSR)
Mice were subcutaneously immunized with 300 μg of BPO-HSA and FCA and spleen cells were obtained 13 days after the immunization. Forty μl of RPMI-1640 medium (Gibco) containing 5 × 10⁷ spleen cells and 40 μg of BPO 51-BGG was intradermally transferred into the left footpad and the FSR was measured 24 hours after the challenge.

Treatment of Spleen Cells by Anti-Thy-1,2 Serum
Anti-Thy-1,2 serum was purchased from Olac 1976 Ltd., Bicester, England and its cytotoxic titer for thymocytes was 500,000. Spleen cells were suspended at 5 × 10⁷ cells/ml in RPMI 1640 medium, and added 1/10 volume of 100-fold diluted anti-Thy-1,2 serum and 1/10 volume of guinea pig serum absorbed by red blood cells and spleen cells, and the cell suspension was incubated at 37°C for 45 minutes.

Desensitization of the Footpad Swelling Reaction (FSR)
One mg of penicilloyl-BGG saline solutions were intravenously injected to the mice immunized with the penicilloyl-HSA and FCA 14 days before. Soon after the i.v. antigen-administration, mice were challenged with penicilloyl-BGG.

Induction of Suppression of Hapten-specific DTH and its Sensitivity for Treatment by Cyclophosphamide
Suppression of hapten-specific DTH was induced by intravenous administration of 300 μg of HSA
a week before the immunization of BPO-HSA and FCA. Treatment of mice by cyclophosphamide was performed by intraperitoneal administration of 150 mg/kg of cyclophosphamide 4 days after the induction of suppression of DTH.

Results

Effect of Various Factors on the Induction of Footpad Swelling Reaction (FSR)

A/J mice were subcutaneously immunized with BPO-HSA and FCA and challenged into footpads with 25 μg of BPO_{31}-BGG on day 11 after the immunization. FSR was measured 24 hours after the antigen challenge. The optimal dose of BPO_{30}-HSA in immunization was 300 μg/mouse, and more than 15 moles of BPO groups were necessary to be bound per mole of HSA for induction of the strong FSR. C57BL/6 and C3H/He mice were high responders, A/J mice moderate, and BALB/c and DBA/2 mice were low responders. In this study A/J mice were routinely used and 300 μg of BPO_{30}-HSA was used for immunization (Table 1).

Effect of Various Factors on Footpad Swelling Reaction (FSR)

Mice were subcutaneously immunized with 300 μg of BPO_{30}-HSA and FCA and challenged into footpads with 0.04 ml of BPO-BGG saline solution. FSR was measured 24 hours after the challenge.

Table 1. Effect of various factors on the induction of footpad swelling reaction for BPO.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Dose of immunogenb)</th>
<th>Epitope densityc)</th>
<th>Strain of moused)</th>
<th>Footpad swelling (x10^{-2} mm)(^e))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 μg/mouse</td>
<td>30</td>
<td>C57B1/6</td>
<td>38±13(^a))</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>60</td>
<td>C3H/He</td>
<td>85±20(^a))</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>75</td>
<td>A/J</td>
<td>142±17(^a))</td>
</tr>
<tr>
<td></td>
<td>3,000</td>
<td>167</td>
<td>BALB/c</td>
<td>39± 8(^a))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DBA/2</td>
<td>138±16(^a))</td>
</tr>
</tbody>
</table>

\(^a)\) Mice were s.c. immunized with BPO-HSA and FCA and challenged into footpads with 25 μg of BPO_{31}-BGG 11 days after the immunization. Footpad swelling reaction was measured 24 hours after the challenge.

\(^b)\) Mice were immunized with indicated dose of BPO_{30}-HSA.

\(^c)\) Mice were immunized with 300 μg of BPO_{n}-HSA.

\(^d)\) Mean±SE of 6 mice.

Table 2. Effect of various factors on footpad swelling reaction.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Dose of antigenb)</th>
<th>Epitope densityc)</th>
<th>Hapten specificityd)</th>
<th>Footpad swelling (x10^{-2} mm)(^e))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.39 μg/mouse</td>
<td>BPO_{31}-BGG</td>
<td>BPO_{31}-BGG (25 μg)</td>
<td>28± 6(^a))</td>
</tr>
<tr>
<td></td>
<td>1.56</td>
<td>BPO_{30}-BGG</td>
<td>BPO_{30}-BGG</td>
<td>55±5(^a))</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>BPO_{18}-BGG</td>
<td>BPO_{18}-BGG</td>
<td>82±5(^a))</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>BPO_{30}-BGG</td>
<td>BPO_{30}-BGG</td>
<td>98±14(^a))</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>BPO_{31}-BGG</td>
<td>BPO_{31}-BGG</td>
<td>94±9(^a))</td>
</tr>
</tbody>
</table>

\(^a)\) Mice were s.c. immunized with 300 μg of BPO_{30}-HSA and FCA and challenged into footpads with BPO-BGGs. Footpad swelling reaction was measured 24 hours after the challenge.

\(^b)\) Mice were challenged with the indicated dose of BPO_{31}-BGG.

\(^c)\) Mice were challenged with 25 μg of BPO_{30}-BGGs.

\(^d)\) Mice were challenged with the indicated dose of antigen or hapten.

\(^e)\) Mean±SE of 6 mice.
Concerning the dose of challenging antigen, more than 6.25 \( \mu \)g of BPO\(_{31}\)-BGG was necessary for induction of strong FSR. More than 30 of epitope density (BPO moles/mole BGG) was necessary for induction of strong FSR, too. In this study 25 \( \mu \)g of BPO\(_{31}\)-BGG was routinely used for the challenge. Twenty five \( \mu \)g of BPO\(_{31}\)-BGG could elicit the FSR. Twenty five \( \mu \)g of HSA slightly elicit the FSR. This FSR was specific for the BPO group (Table 2).

Kinetics of Induction of Footpad Swelling Reaction (FSR) and Antibody Response

FSR could be induced on day 4 and reached the maximum on day 11~14 after the immunization. Thereafter, FSR decreased gradually and was very weak on day 30 after the immunization. 2-Mercaptoethanol (2-ME) sensitive antibodies (IgM) for BPO were detected 2 weeks after the immunization and total antibodies for BPO reached the maximum 3 weeks after the immunization. 2-ME resistant

Fig. 1. Kinetics of induction of footpad swelling reaction and antibody response.

Mice were immunized with 300 \( \mu \)g of BPO\(_{30}\)-HSA and FCA and challenged with 25 \( \mu \)g of BPO\(_{31}\)-BGG at various times after immunization.

Each point and vertical bar represent the mean and SE of 5 mice, respectively.

Fig. 2. Kinetics of footpad swelling reaction.

Mice were immunized with 300 \( \mu \)g of BPO\(_{30}\)-HSA and challenged with 25 \( \mu \)g of BPO\(_{31}\)-BGG 12 days after immunization. Footpad swelling reaction was measured at various times after challenge.

Each point and vertical bar represent the mean and SE of 5 mice.

Table 3. Transfer of footpad swelling reaction.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Treatment of transferred cell</th>
<th>Footpad swelling (x10^-3 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor( ^a )</td>
<td>—</td>
<td>89±7( ^c )</td>
</tr>
<tr>
<td>Recipient( ^b )</td>
<td>C'</td>
<td>74±8</td>
</tr>
<tr>
<td>Recipient</td>
<td>Anti-Thy-1,2+C'</td>
<td>29±2</td>
</tr>
</tbody>
</table>

\( ^a \) Mice were immunized with 300 \( \mu \)g of BPO\(_{30}\)-HSA and FCA and challenged into footpads with 25 \( \mu \)g of BPO\(_{31}\)-BGG 12 days after immunization.

\( ^b \) Mice were transferred into footpads with mixture of 25 \( \mu \)g of BPO\(_{31}\)-BGG and BPO\(_{30}\)-HSA primed spleen cells of donor mice. Spleen cells were obtained 12 days after immunization and treated by guinea pig complement or anti-Thy-1,2 and complement.

\( ^c \) Mean±SE of 5 mice.
antibodies (IgG) for BPO was first observed 3 weeks after the immunization and gradually increased up to 5 weeks after the immunization (Fig. 1).

Kinetics of Footpad Swelling Reaction (FSR)
Mice were immunized with BPO-HSA and challenged with BPO-BGG on day 12 after the immunization. The thickness of footpads decreased up to 6 hours after the challenge, then rapidly increased and reached the maximum 18~24 hours after the challenge. Thereafter, the thickness of the footpad gradually decreased (Fig. 2).

Transfer of the Footpad Swelling Reaction (FSR) and its Sensitivity for the Treatment by Anti-Thy-1,2 Serum
A strong FSR was observed in the mice immunized with BPO-HSA and the mice transferred with BPO-HSA-primed spleen cells. But FSR was not observed in the mice transferred with BPO-HSA-primed spleen cells treated with anti-Thy-1,2 serum and complement (Table 3).

Histological Study
Infiltration of monocytes was observed and polymorphonuclear cells were not observed in the footpads 48 hours after the antigen challenge (Fig. 3). But in the footpads 24 hours after the antigen challenge, a few polymorphonuclear cells were observed, and such infiltration of polymorphonuclear cells was usually observed in FSR of mice.

Cross-reactivity of Penicillins in DTH
Various penicilloyl-BGG were challenged to the mice immunized with penicilloyl-HSA; BPO, ABPO and SBPO groups cross-reacted with each other and the FPO group cross-reacted even more with BPO, ABPO and SBPO groups. When more than 100 µg of penicilloyl-BGG was intravenously injected to the mice just before the antigen challenge, FSR was almost inhibited. Then, mice were intravenously injected with 1 mg of various penicilloyl-BGGS just before the antigen challenge corresponding to the immunized antigen. BPO-, ABPO- and SBPO-BGGS could inhibit the FSRs for BPO, ABPO and SBPO. And BPO-, ABPO- and SBPO-BGGS could inhibit the FSR for FPO as well as FPO-BGG. The common cross-reactivity was observed in FSR and its desensitization (Table 4).

Induction of Suppression of Hapten-specific DTH and its Sensitivity for Treatment by Cyclophosphamide
The FSR for BPO was suppressed by the preadministration of carrier protein (HSA).
Discussion

FSR in mice was examined for the purpose of induction of DTH for penicillin. The studies of various factors in immunization and the FSR resulted in the constant and strong FSR for penicillin. Kinetics and histological study of the FSR showed that this FSR for penicillin was the typical DTH.

Even more, the FSR can be transferred to normal syngenic mice by the antigen-primed spleen cells and not by the anti-Thy-1,2 serum treated cells. These results showed that the FSR for penicillin in this study was the cell-mediated immunity: DTH. Cross-reactivity of penicillins in DTH was then studied.

The DTH for the penicilloyl group contained the DTH for the common penicilloyl moiety of penicillin. The role of the acyl side chain moiety in the cross-reactivity of penicillin in DTH was difficult to be proved because of this common cross-reactivity. However, such cross-reactivity of penicillin was due to the acyl side chain moiety and the penicilloyl moiety in rabbit anti-penicilloyl IgG antibodies but only due to the acyl side chain moiety in rat anti-penicilloyl IgE antibodies. Rabbit antipenicilloyl IgG antibodies contain the specificity for the penicilloyl moiety but rat antipenicilloyl IgE antibodies did not. The FSR for penicillin was likely to disappear in proportion to the IgG antibodies for penicillin. This decrease of FSR was thought to be caused by the cells associated with the antibody production system, but the circulating antibodies may cause this decrease of FSR by eliminating the challenged antigen in the footpad. Pro-

Table 4. Cross reactions of penicillins.

1) In footpad swelling reaction.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Challenged antigen</th>
<th>BPO-BGG</th>
<th>ABPO-BGG</th>
<th>SBPO-BGG</th>
<th>FPO-BGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPO-HSA</td>
<td>100b)</td>
<td>58</td>
<td>74</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>ABPO-HSA</td>
<td>97</td>
<td>100</td>
<td>80</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>SBPO-HSA</td>
<td>85</td>
<td>62</td>
<td>100</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>FPO-HSA</td>
<td>117</td>
<td>95</td>
<td>93</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

2) In desensitization of footpad swelling reaction.

<table>
<thead>
<tr>
<th>Systemc)</th>
<th>Desensitizing antigen</th>
<th>BPO-BGG</th>
<th>ABPO-BGG</th>
<th>SBPO-BGG</th>
<th>FPO-BGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPO</td>
<td>100e)</td>
<td>82</td>
<td>84</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>ABPO</td>
<td>93</td>
<td>100</td>
<td>88</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>SBPO</td>
<td>70</td>
<td>58</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>FPO</td>
<td>75</td>
<td>72</td>
<td>61</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Mice were immunized with 300 μg of penicilloyl-HSA and challenged with 25 μg of various penicilloyl-BGGs 11 days after immunization.

Percent reactions against footpad swelling reaction of homologous system. Absolute values of footpad swelling reactions of homologous systems of BPO, ABPO, SBPO and FPO were 101, 130, 128 and 78×10⁻² mm as mean of 6 mice, respectively.

Mice were immunized with penicilloyl-HSA and FCA and 11 days after, i.v. injected with 1 mg of various penicilloyl-BGGs. Soon after the i.v. injection of penicilloyl-BGG, mice were challenged into footpads with penicilloyl-BGG corresponding to the immunogen.

The same hapten conjugated BGG was challenged into footpad as immunized hapten-HSA.

Percent desensitization against desensitization of homologous system. Absolute values of % desensitization of BPO, ABPO, SBPO and FPO homologous systems were 96, 98, 84 and 95 %, respectively.

Table 5. Effect of cyclophosphamide on suppression of hapten specific footpad swelling reaction by i.v. administration of carrier protein.

<table>
<thead>
<tr>
<th>Administration of HSA</th>
<th>Treatment of CPA</th>
<th>Footpad swelling (x10⁻² mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>No</td>
<td>106±10e)</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>42±7</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>84±9</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>124±5</td>
</tr>
</tbody>
</table>

Mice were i.v. administered with 300 μg of HSA on day 0, i.p. administered with 150 mg/kg of CPA on day 4 and immunized with BPO-HSA on day 7. Footpad swelling reaction was measured 14 days after immunization.

a) Mean±SE of 5 mice.

This suppression was sensitive for administration of cyclophosphamide. And the administration of cyclophosphamide slightly enhanced the FSR in normal mice (Table 5).

Discussion

FSR in mice was examined for the purpose of induction of DTH for penicillin. The studies of various factors in immunization and the FSR resulted in the constant and strong FSR for penicillin. Kinetics and histological study of the FSR showed that this FSR for penicillin was the typical DTH. Even more, the FSR can be transferred to normal syngenic mice by the antigen-primed spleen cells and not by the anti-Thy-1,2 serum treated cells. These results showed that the FSR for penicillin in this study was the cell-mediated immunity: DTH. Cross-reactivity of penicillins in DTH was then studied. The DTH for the penicilloyl group contained the DTH for the common penicilloyl moiety of penicillin. The role of the acyl side chain moiety in the cross-reactivity of penicillin in DTH was difficult to be proved because of this common cross-reactivity. However, such cross-reactivity of penicillin in DTH seems to resemble the cross-reactivity in the IgG antibodies rather than the IgE antibodies. Namely, the cross-reactivity of penicillin was due to the acyl side chain moiety and the penicilloyl moiety in rabbit anti-penicilloyl IgG antibodies but only due to the acyl side chain moiety in rat anti-penicilloyl IgE antibodies. Rabbit antipenicilloyl IgG antibodies contain the specificity for the penicilloyl moiety but rat antipenicilloyl IgE antibodies did not. The FSR for penicillin was likely to disappear in proportion to the IgG antibodies for penicillin. This decrease of FSR was thought to be caused by the cells associated with the antibody production system, but the circulating antibodies may cause this decrease of FSR by eliminating the challenged antigen in the footpad.
bably, the ARTHUS reaction was enhanced when the circulating antibodies were produced. In our preliminary experiment we observed that the suppression of FSR was transferred to the mice immunized 2 weeks before by the antigen-primed spleen cells obtained 5 weeks after the immunization. Therefore it is felt that the DTH suppressor cells exist in the cells resulting to antibody production system. Relative to the suppression of DTH, intravenous administration of antigen was often used and the induction of suppressor cells of DTH in this procedure was attempted. The intravenous preadministration of carrier protein HSA could induce the suppressor of the hapten-specific DTH since the effect of intravenous preadministration of carrier protein disappeared by the treatment of cyclophosphamide.

Acknowledgment

The authors wish to thank T. Yusa for his technical assistance.

References