Development of a Penicillin-reactive IgE Screening Platform

I-Ju Chang\textsuperscript{a}, Chia-Rui Shen\textsuperscript{a}, Wen-Hung Chung\textsuperscript{b}, Chao-Lin Liu\textsuperscript{c}, Chuan-Chian Chiou\textsuperscript{a}

\textsuperscript{a}Graduate Institute and Department of Medical Biotechnology and Laboratory Science, Chang Gung University, Kweishan, Taiwan.

\textsuperscript{b}Department of Dermatology, Chang Gung Memorial Hospital, Kweishan, Taiwan.

\textsuperscript{c}Graduate School of Biochemical Engineering, Min Chi University of Technology, Taishan, Taiwan.

\textbf{Abbreviations:} OVA: ovalbumin; ELISA: enzyme-link immunosorbent assay

Corresponding author: Chuan-Chian Chiou

Graduate Institute and Department of Medical Biotechnology and Laboratory Science, Chang Gung University, 259 Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan 333, Taiwan.

Phone: +886 32118800 ext.5202

Fax: +886 32118698

Email: ccchiou@mail.cgu.edu.tw
Abstract

Penicillins are effective for treating bacterial infections in many clinical situations, but in certain cases, they cause allergic reactions varying from mild skin rashes to anaphylactic shock and death. These symptoms often resemble those seen in immediate or delayed hypersensitivity reactions, and may be associated with drug specific IgE and/or drug specific T cells. Currently, the laboratory tests include isotope based serum IgE antibody measurement as well as skin test. However, radioisotope allergen specific tests (RAST) are not convenient, and the standardized skin-testing reagents and protocol do not usually exist.

In fact, the skin test is often avoided because of the high risks. Therefore, in the current study, we have developed a non-isotope based penicillin-reactive IgE screening platform with high sensitivity and specificity. Using this screening platform to detect IgE of a mouse model sensitized with penicillin reveals that high levels of penicillin specific IgE were found in the penicillin sensitized animals but not in OVA sensitized or untreated control animals. Penicillin specific T cells produce relatively higher levels of IL-4 and IL-5 in the penicillin sensitized group, indicating a Th2-cytokine dominated response of T-cell dependent IgE production against penicillin. This observation and the development of such convenient platform to measure penicillin specific IgE may benefit further study of penicillin allergy.

Key words: animal model, drug-allergy, ELISA, immunoglobulin (Ig)-E, penicillin
Introduction

Allergic drug reactions demonstrate characteristics that are common to the immunologic reactions in many other allergies, including a range of responses from a mild rash to life-threatening effects on many body systems. Penicillin is used widely to treat clinical infection and to prevent bacterial contamination of tissue culture. However, allergic reactions to penicillin are the most common cause of immunologic drug reactions. Penicillin hypersensitivity may produce many different clinical symptoms. These symptoms often resemble those seen in immediate or delayed hypersensitivity reactions, and may be associated with drug specific IgE and/or drug specific T cells (de Haan & van Ketel, 1987). Anaphylaxis, appearing within one hour after exposure to penicillin, represents the most severe type of allergy to penicillin. Hypotension and death may occur without any other symptoms although pruritus, urticaria and angioedema, laryngeal oedema and, more rarely, rhinitis and asthma, may also appear. One to 72 hours after exposure of a sensitized individual to penicillin antigen accelerated allergic reactions can sometimes be seen. Late adverse reactions are sometimes seen more than 3 days after penicillin administration. Such reactions include skin eruptions such as exanthemas on the trunk and proximal extremities (Ahlstedt, 1984).

To understand why penicillin allergy might develop, it is important to consider how the penicillin-derived immunogens are formed. Penicillin is a low molecular weight substance
which needs to combine with protein to become immunogenic. Its β-lactam ring is a target for nucleophilic attack by free amino groups of proteins, leading to ring opening and covalent amide bonding of the penicilloyl group (Bachelor, 1965). The penicilloyl configuration, where the hapten determinant is covalently linked to ε-amino groups of lysine residues of proteins, constitutes more than 90% of the reaction products between proteins. In this form they act as haptens that can be recognised by MHC-restricted T cells and IgE (Blanca, 1995) and also referred to as the major antigenic determinant for other antibodies. Thus, it appears that both T and B cells are involved in specific recognition of penicillin derived epitopes (Pichler & Yawalkar, 2000). In fact, the penicilloyl group plays a role in 75% of IgE mediated allergic reactions and is the target for most other types of hypersensitivity reactions, including delayed type hypersensitivity (Sullivan & Weiss, 1993).

T helper cells, CD4+, can be further classified into two sub-groups, T helper 1 (Th1) and T helper 2 (Th2), according to the cytokines they secrete (Paul & Seder, 1994). The functional Th1/Th2 dichotomy is likely to play a role also in the allergic phenomena induced by penicillins, in particular with a prevalence of Th2 responses in immediate hypersensitivity reactions and of Th1 responses in delayed allergic phenomena. Evidence for this has been found by measuring the cytokine profiles of T cell lines from the peripheral blood of patients with penicillin-induced exanthems. Additionally, cell lines from subjects suffering from bullous exanthems, one of the clinical features of delayed type reactions, secreted high levels
of IFN-γ, whereas cells from patients with drug-induced urticaria produced IL-4 (Merk & Hertl, 1996).

Penicillin allergy may cause allergic reactions varying from mild skin rashes to anaphylactic shock and death. The current diagnosis mainly depends on the skin test, which is often avoided because of the high risks. Since the symptoms of penicillin allergy often resemble those seen in immediate or delayed hypersensitivity reactions, and associated with drug specific IgE and/or drug specific T cells. It is well known that ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample; it is a useful tool both for determining serum antibody concentrations and also for detecting the presence of antigen (Engvall & Perlmann, 1972). The ELISA seems to be more sensitive for the detection of anti-penicillin antibodies IgE than the RAST (de Haan, Boorsma, & Kalsbeek, 1979). Therefore, in the current study, we aimed to develop a sensitive and specific ELISA screening platform to detect penicillin-reactive IgE. Moreover, a mouse model representing penicillin allergy were also established and utilized to study the penicillin specific T cells and their responses.
Materials and methods

Animals

BALB/c mice were obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and maintained at the animal facility of Chung Gung University in accordance with the institutional animal care protocol. All of the animal studies were approved by the animal committee of the Chang Gung University. Mice utilized in this study were female and aged 6 weeks and allowed to rest for a few days before use to overcome travel-induced stress.

Antibiotic-protein conjugates

Conjugates (penicillin-transferrin, penicillin -OVA) were prepared by the method of de Haan with some modification (de Haan et al., 1979). Briefly, 300 mg of penicillin G was added to a solution of 100 mg candidate carrier proteins (such as transferrin and OVA) in 10 ml of distilled water, and then adjusted the pH at 10 with NaOH. The resultant solution was then dialyzed for 2 days against 0.01 M PBS (pH 7.2), and their aliquots (10 mg/ml) were stored at -30°C.

Dot blot

After methanol activation, PVDF membrane was loaded with different dilutions of
penicillin conjugate samples, and followed by blocking with 3% BSA in PBS and detecting with mouse antiserum specific for penicillin. The blot were visualized using the enhance chemiluminescence system (ECL, Amersham Biosciences).

Allergen sensitization

Groups of 6-8 weeks BALB/c mice were sensitized by four intraperitoneal (i.p.) injections of 50 µg OVA-penicillin conjugates which had been emulsified in 0.8 mg aluminium hydroxide in 200 µl of saline on day 1, 2, 3 and 13. Normal saline control mice were injected with 200 µl of saline or 50 µg OVA emulsified in 0.8 mg aluminium hydroxide in 200 µl of saline. Allergen challenge was made on days 13, 16, 20, 23 and 26 by inhaling mice with either OVA aerosols or normal saline (normal saline controls) group) or (OVA group and penicillin-OVA group).

Enzyme-link immunosorbent assay (ELISA)

The presence of penicillin-specific antibodies in sera was determined by ELISA. Briefly, 50 µl of 10 µg/ml of penicillin-transferrin, transferrin only as controls, were coated in 96-well microtiter plates with 0.1 M carbonate buffer pH 9.6 by overnight incubation at 4 °C. Coated plates were washed twice with 0.05 % tween 20 in PBS and then blocked with 1% bovine serum albumin (Sigma) in PBS at room temperature for 2 hours. Diluted sera were
applied to wells at room temperature for 1 hour. Following the addition of biotin-conjugated rat anti-mouse IgE, IgG1 or IgG2a, and streptavidin-conjugated HRP, the assay was developed with 3,3’5,5’-tetramethylbenzidine (TMB). Termination of the reaction was achieved by adding 50 µL/well 2N sulphuric acid (H₂SO₄). The plates were read at 450 nm using an ELISA plate reader (Molecular Devices, CA, USA).

Preparation of lymphoid cells

Tissues were suspended and dissected out in Hank’s balanced salt solution (HBSS, GibcoBRL) and minced through 100 mm stainless steel screen mesh in a petri dish (Falcon). The resultant cell suspension was gently resuspended in order to disaggregate any cell clumps before transfer to clean centrifuge tube (Falcon, Becton Dickinson Labware, New Jersey, USA). The debris was allowed to sediment, and the cell suspension was then decanted to a fresh centrifuge tube and washed three times with the centrifugation at 1500 rpm for 5 minutes in a bench centrifuge.

Cytokine production from antigen stimulated splenic T cells

Spleens of individual mice were obtained from normal and sensitized mice sacrificed by cervical dislocation, and single cell suspension was made by mincing through 100 mm stainless steel screen mesh. Splenic T cells were enriched by passing the spleen cells through
the Nylon wool (PerkinElmer Life Sciences, Inc.) column by gravity flow. Cell cultures, consisting of $1 \times 10^6$ cells/ml fresh prepared splenic T cells were stimulated with/without penicillin G, transferrin, penicillin-transferrin and OVA protein at final concentration of 10µg/ml. Con A or medium alone served as positive and background controls, respectively. The complete medium used was MEM medium supplemented with 4 mM L-glutamine (GibcoBRL), 100 µg/ml penicillin/Streptomycin, 20 Mm HEPES (Biochrom), and $5 \times 10^{-5}$ M 2-mercaptoethanol (2-ME) (Sigma) and fresh 5% complement-inactivated (at 56°C for 30 mins) fetal bovine serum (Hyclone). Supernatant was harvested at day 4, 5, 6 and 7 of the culture and assayed for cytokines production.

*Cytokines production assay by ELISA or Cytometric Bead Array (CBA\textsuperscript{BDTM})*

Cytokine produced by antigen stimulating splenic T cells were mainly quantitated by *Cytometric Bead Array* (BD) in accordance with the manufacturer’s instructions. Briefly, one vial of lyophilized Mouse Th1/Th2 Cytokine Standards was reconstituted with Assay Diluent, and the Assay Diluent only was served as negative control. Each Capture Bead suspension was added (6 µL each Capture Beads/sample) into a single tube, and followed by PE detection reagent. After incubating for 2 hours, the supernatant was removed and followed by another wash before analyzed by flow cytometer. Several cytokines including mouse IL-4, IL-5 and IFN-γ were further verified by ELISA using the matching cytokine antibody set.
(R&D Systems, Inc. MN, USA) in accordance with the manufacturer’s instructions.

**Statistical analysis**

The statistical significance of differential findings between experimental groups of animals was determined by Student’s *t* test. Differences were considered statistically significant if the value of *p* was ≤ 0.05.

**Results**

*Conjugation of penicillin with transferrin or OVA proteins by alkaline incubation*

To determine whether penicillin was conjugated successfully with transferrin or OVA proteins, dot blot assay was adapted. Figure 1, A shows that penicillin in both conjugates was able to be detected by mouse antiserum against to penicillin antibodies. In fact, penicillin can be detected through serial dilution until the conjugates were of 50 ng for penicillin-transferrin, and 500ng for penicillin-OVA. Figure 1, B and C show that both conjugates could be verified by either anti-transferrin or OVA antibodies.

*Establishment of an animal model with high titers of antibody IgE against penicillin*

Recipient mice were injected i.p five times with 200 µl OVA (50 µg) or penicillin-OVA. Collected in a time dependent manner followed the course of sensitization
(see Figure 2), and the serum levels of IgE, IgG1 and IgG2a were determined by ELISA. As shown in Figure 3, A that penicillin specific IgE could be detected in a serial diluted range from 20 to 2560 of serum samples obtained from 3 penicillin sensitized mice. In fact, it can be seen from Figure 3, B that significantly higher levels of penicillin specific IgE sera from penicillin-sensitized mice than those from the mock mice, which was sensitized with OVA only. Moreover, penicillin-sensitized mice produce not only penicillin-specific IgE (Figure 4, A), but also significantly higher levels of penicillin-specific IgG1 and relatively higher levels of penicillin-specific IgG2a (Figure 4, B) in contrast to both mock and normal saline controls, indicating that Th2-towards responses against penicillin were activated in these penicillin-sensitized mice. Moreover, similar elicitation of OVA-specific IgE and IgG1 was found in both penicillin sensitized group and the mock controls, whereas a remarkably lower level of OVA-specific IgG2a was seen in pencillin-sensitized group.

_Cytokine profile of splenic T cells responding to penicillin and other antigens_

Splenic T cells derived from mice that i.p injection of penicillin-OVA were stimulated with OVA in cultures and their responses compared to those given by T cells derived from normal saline injection mice. In Figure 6, T cells from OVA or penicillin-OVA sensitized mice produced similar higher levels of IL-4, IL-5 and IFN-g in contrast to those in normal saline controls. In the presence of OVA, a appreciably increase in IL-4 and IL-5
concentration was produced by T cell derived from mice i.p injected of penicillin-OVA and the mock control mice i.p injected of OVA. It is noted that, except the group of mice receiving OVA sensitization, the stimulation of OVA enhanced the mice received normal saline treatment or penicillin sensitization to produce IFN-g, suggesting that OVA directly affect the responses of T cells derived from OVA-sensitized mice but, perhaps, not from penicillin-sensitized mice.
People using the similar protocol of OVA sensitization did show that OVA challenge was able to evoke the higher OVA-specific Th2 responses including high levels of serum IgG1 and IgE antibodies (Wu, Chen, & Kuo, 2006). In fact, the level of IgE in serum usually reflexes the severity of anaphylaxis, as immediate hypersensitivity is mainly mediated by IgE. When the allergen binds the IgE FcRI complex of the mast cells or basophils, the cells then release histamine and other various inflammatory mediators. In the allergic asthma, these inflammatory mediators will further induce contraction of the smooth muscle of the ileum, bronchi and bronchioles and increase vascular permeability. In this study, I have utilized the OVA sensitization to induce high levels of OVA-specific IgE response and moreover, the penicillin specific IgE can also be induced in the penicillin-OVA conjugate-sensitized mice.

In fact, it may be argued whether the IgE response to penicillin is antigen-specific in the penicillin-OVA conjugate-sensitized mice. It is shown from the figure 2 that significantly higher levels of IgE against only penicillin-transferrin conjugates but not transferrin alone can be seen in the penicillin-OVA conjugate-sensitized mice. Moreover, lower antibody response to penicillin alone was obtained, as penicillin is considered as a hapten and is not able to evoke immune responses without conjugating to carrier proteins.

It is known that the production of both IgE and IgG1 is Th2-mediated responses. Indeed,
in the penicillin-OVA conjugate-sensitized mice, not only the penicillin specific IgE antibodies but also IgG1 were induced. In fact, we have been trying to identify the cytokine profile of the splenic T cells responding to penicillin in the penicillin-OVA conjugate-sensitized mice. We predict that both penicillin and OVA-responding T cells should produce more Th2-associated cytokines, such as IL-4 and IL-5. However, the current findings are not satisfactory. It may result from the inappropriate collecting protocol or the not-good storage. Or we need more tests to confirm the result.

Additionally, most cytokine responses were elicited in the day 6 of antigen-stimulated cultures, it reveals the general secondary response to a foreign antigen. If it is a recall antigen stimulation, the cytokines should be detected on the day 4 or 5 of cultures. Moreover, there seems to be cytokine responses including IL-2, 4, 5 and IFN-g to OVA among the whole culture supernatants. It is assumed that the OVA used in the stimulation may be contaminated or dissolved badly. Finally, the transferrin used in the cultures stimulated some TNF response, indicating that some bacterial contaminants within the antigen preparations.

In conclusion, although penicillin now is not a common use in most clinical situations, its allergic reactions particular anaphylactic shock and death still remain a crucial issue. The observations and establishments of the diagnostic platform as well as an animal model will benefit for further investigation of penicillin and other antibiotic allergy.
Acknowledgments

The study is financially supported in part by Chang Gung University Grant EMRPD160221 (Ministry of Education, Taiwan) to CCC and CRS, and National Science Council grant 96-2221-E-131-008 to CLL.

References


Figure legends

Figure 1. Penicillin detection in penicillin-protein conjugates
Serial dilutions of transferrin and penicillin-transferrin, OVA and penicillin-OVA as dictated were prepared for penicillin detection by dot blotting. The detecting antibodies utilized (A) mouse antiserum specific for penicillin, (B) goat antiserum specific for transferrin and (C) mouse antiserum specific for OVA.

Figure 2. Schematic diagram depicting protocols for penicillin-OVA induced allergy protocol
Mice sensitized and challenged with penicillin-OVA conjugates as described in Materials and methods. N.S = normal saline; OVA = ovalbumin

Figure 3. Abundant penicillin-specific IgE in Penicillin sensitized mice
The level of penicillin specific antibody IgE was determined by ELISA. Multiwell plates were coated with penicillin-transferrin and followed by blocking with 1 % BSA. (A) serum samples obtained from penicillin-sensitized mice or mock control (see method) were serial diluted, incubated and detected by anti-mouse IgE. (B) High serum titers of penicillin specific antibody IgE in penicillin sensitized mice. Data are presented as mean ± SEM.

Figure 4. Serum levels of penicillin-specific antibodies in penicillin sensitized mice
The level of penicillin (-transferrin) or transferrin specific antibodies including (A) IgE, (B) IgG1 and IgG2a were determined by ELISA. Data are presented as mean ± SEM. Group 1: normal saline controls (n=5); Group 2: mock (OVA only) controls (n=7); Group 3 = penicillin-sensitized group (n=6)
Figure 5. Serum levels of OVA-specific antibodies

The level of OVA-specific antibodies were determined with ELISA. Briefly, 96-well plate were coated with OVA. After blocking with 1 % BSA, serum samples added into the wells and followed by the detection antibody anti-mouse IgE(A), IgG1 and IgG2a (B). Data are presented as mean ± SEM. Group 1: normal saline controls (n=5); Group 2: mock (OVA only) controls (n=7); Group 3= penicillin-sensitized group (n=6)

Figure 6. IL-4, IL-5 and IFN-g production of splenic T-cells responding to antigens.

BALB/C mice at 6-8 weeks of age were sensitized with penicillin-OVA or normal saline. Twenty-seven days after the first treatment, splenic T cells were isolated and stimulated with different antigens. On day 6 of antigen stimulation in vitro culture, culture supernatant was harvested and analyzed for the cytokine profile by CBA method. Data are presented as mean ± SEM. Group 1: normal saline controls (n=2); Group 2: mock (OVA only) controls (n=3); Group 3= penicillin-sensitized group (n=3)
Fig. 1

(A)

(B)

(C)
Fig. 2

N/S or Penicillin-OVA/alum (i.p.)  N/S or OVA aerosols

Day 0 1 2 3 9 13 16 18 20 23 26 28

bleeding

Sacrifice
Fig. 3

(A) 

Mock penicillin-sensitized (2X X10)^-1 dilution

(B) 

Mock penicillin-sensitized

O.D 450
Fig. 4

(A) IgE

![IgE graph](image)

(B) IgG1 and IgG2a

![IgG1 and IgG2a graph](image)
Fig. 5

(A) OVA specific IgE

(B) IgG1 and IgG2a
Fig. 6

(A) IL-4

(B) IL-5

(C) IFN-g