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Research article

Establishment of a basophil activation test in BN rats

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Abstract: Allergies induced by low molecular weight compounds (LMWCs) have become a major problem in clinical medication. Effective preclinical prediction of sensitization can solve this problem; however, there are currently no reliable methods to predict sensitization, and it is necessary to establish a more effective model. The basophil activation test (BAT) has been widely used clinically to diagnose and research allergic diseases with high sensitivity. The purpose of this study was to detect the expression of CD63 in basophils in OVA-sensitized brown Norway (BN) rats, establishing a model for the BAT to assess the sensitization of the low molecular weight compound PG. The number of basophils in rats was detected by flow cytometry and blood smear analysis. The peripheral blood of OVA-sensitized rats was incubated with OVA, and the increase in CD63 on basophils was greater than that with PBS in vitro. After penicillin G-conjugated OVA (PG/OVA) was incubated with the peripheral blood of penicillin G-conjugated BSA (PG/BSA)-sensitized rats, the expression of CD63 on basophils was significantly higher than that with PBS. The basophils in the sensitized rats were again exposed to the same antigen in vitro, and flow cytometry detected a significant increase in CD63, demonstrating that the rat BAT model was successfully built, and it can be used to evaluate the potential sensitization of low molecular weight compounds.

Keywords: basophil activation test; CD63; low molecular weight compounds; immediate hypersensitivity reactions; safety evaluation in preclinical

1. Background

Immediate hypersensitivity reactions (IHRs) are induced by the immunogenicity of a drug itself, drug metabolites or dressings. The main clinical manifestations of IHRs are dermatitis, urticaria,

asthma, angioneurotic oedema, gastrointestinal allergy, anaphylactic shock and death [1,2]. Therefore, evaluation of the potential immunosensitization of candidate compounds is essential for the study and development of new drugs. However, due to the lack of ideal preclinical experimental models, it is currently impossible to accurately predict the potential immunotoxicity of drugs, especially the sensitization of low-molecular-weight compounds (LMWCs), which is a global problem. To improve the ability to predict the sensitization of LMWC, researchers must establish a more effective and novel model.

The basophil activation test (BAT) has been widely used in the clinic and shows high sensitivity in the diagnosis of allergic diseases [3,4]. Evidence has shown that the mechanisms of IHRs in brown Norway (BN) rats are similar to those in humans, so BN rats are widely used as drug allergy models for immunological studies domestically and abroad [5,6]. Based on these findings, the aim of this project was to establish a suitable model for the BAT to predict the potential immunosensitization of LMWCs. First, identification of the basophil population in the peripheral blood of rats was necessary. Second, the expression of CD63, an activation marker for rat basophils, was detected by flow cytometry. Finally, the mechanism of PG-induced IHR was simulated, and the blood of penicillin G-conjugated BSA (PG/BSA)-sensitized BN rats was incubated with penicillin G-conjugated OVA (PG/OVA) in vitro. The expression of CD63 was observed to verify that the BAT model was effective.

2. Materials and Methods

2.1. Animals

SPF 6- to 8-week-old BN rats weighing 160–200 g were purchased from Beijing Wei Tong Li Hua Experimental Animal Technology Company (Animal Certificate № 11400700176123). All animals were killed by carbon dioxide after the project was completed.

2.2. Reagents

Penicillin G-conjugated OVA (PG/OVA), penicillin G-conjugated BSA (PG/BSA) (China Bioss), ovalbumin (OVA) (USA Sigma), anti-rat CD45 APC (USA Biolegend), rat IgE (heavy chain) [marker-1] (FITC) (USA GenTex), PE rat anti-mouse CD63, (Germany Miltenyi), Evans blue (USA Sigma) and pentobarbital sodium (Germany Merck) were obtained.

2.3. Sensitization of rats with ovalbumin

BN rats were subcutaneously injected with 1 ml of 1 mg/ml OVA solution (0.5 ml $Al(OH)_3$) on the 1st, 3rd and 5th days. In the control group, normal saline (NS) was used instead of OVA.

2.4. Identification of rat basophils by flow cytometry

Blood was taken on the 10th day after the last sensitization, and blood samples were incubated and analysed within 8 h. 100 μl peripheral heparinized blood was added to 1 μl of anti-rat CD45 APC and rat IgE and stained in the dark for 30 min at 4 °C. 2 ml of ammonium chloride lysate was added,

lysed at room temperature for 10 min and centrifuged for 5 min at 1500 r/min, and the supernatant was removed and lysed 2 times. The cells were washed 2 times with PBS. The cells were resuspended in 100 μl of PBS before testing. Originally, the typical leukocyte region was gated on forward and side scatter (approximately 100 000). Basophil populations were identified as CD45 APC^{low}/IgE $FITC^{high}$ cells.

2.5. Establishment of the basophil activation test

2.5.1. Passive cutaneous anaphylaxis

Antiserum preparation: On the 10th day after the last sensitization, the blood of sensitized rats was taken to prepare serum and stored at −20 °C.

Passive cutaneous sensitization: The 10 normal rats were randomly divided into 2 groups and anaesthetized with 45 mg/kg pentobarbital sodium by intraperitoneal injection. The fur on the back of the rats was removed from an approximately 3 cm × 4 cm region. The antiserum prepared in each group was diluted 1:2, 1:8, and 1:32, and 0.1 ml from each group of antiserum was intradermally injected into the area with no fur on the rats.

Passive cutaneous anaphylaxis challenge: After 24 h of passive cutaneous sensitization, the rats in each group were injected with 1.0 ml of OVA (1 mg/ml) and 1% Evans blue.

Results: The rats were sacrificed after 30 min of challenge, and the dorsal skin was removed to measure the size of the blue spots. Those with a diameter greater than 5.0 mm were judged to be positive. Irregular spots were half the diameter of the sum of the major and minor diameters.

2.5.2. Basophil activation test

Heparin complete anticoagulation blood (200 μl) was added to 2 tubes, each 100 μl. One tube was added to 10 μl of PBS, and the other was added to 10 μl of OVA (10 μg/ml), incubated at 37°C for 15 min, and placed on ice for 5 min, and degranulation was stopped. Then, 1 μl each of anti-rat CD45 APC, rat IgE and PE rat anti-mouse CD63 was added and stained for 30 min at 4 °C in the dark. Then, 2 ml of ammonium chloride lysate was added, and the sample was lysed for 10 min at room temperature and centrifuged at 1500 r/min for 5 min. The supernatant was removed and lysed 2 times. The cells were washed with PBS 2 times. The cells were resuspended in 100 μl of PBS. First, the typical leukocyte population on the forward and side scatter was gated (approximately 100 000), and $CD45^{low}$/IgE^{high}-labelled cells were identified as basophils. The expression of CD63 was measured after basophils were stimulated in vitro.

2.6. Verification of the rat basophil activation test

2.6.1. Sensitization of rats with PG/BSA

BN rats were subcutaneously injected with 1 ml of 1 mg/ml PG/BSA (0.5 ml of $Al(OH)_3$) solution on the 1st, 3rd and 5th days. In the control group, NS was used instead of PG/BSA.

2.6.2. Passive cutaneous anaphylaxis

The method of passive cutaneous anaphylaxis (PCA) was the same as in 2.5.1. Twenty-four hours after passive cutaneous sensitization, the rats in the control group and the PG/BSA-sensitized group were injected with NS and PG/OVA (1 mg/ml) plus 1% Evans blue, respectively.

2.6.3. Basophil activation test

Complete blood with the heparin anticoagulant (200 μl) was added to 2 tubes, each 100 μl. One tube was added to 10 μl of PBS, and the other was added to 10 μl of PG/OVA (10 μg/ml). The method of flow cytometry detection was the same as in 2.5.2.

2.7. Ethics approval of research

All animal experimental procedures and protocols of the study were approved by the Ethics Committee for Animal Experiments of the Shandong University Institute of Preventive Medicine (Permit Number: 20111231) in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.8. Statistical analysis

With GraphPad Prism 5 for Windows statistical software, the numbers of basophils and CD63, after the data conformed to the normal distribution, expressed as x ± s, were compared using two independent samples t tests, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of rat basophils

3.1.1. Identification of rat basophils by flow cytometry

Flow cytometry showed that the CD45 APC^{low}/IgE $FITC^{high}$-labelled rat basophils were grouped together (Figure 1a,b). The percentages of basophils in the control group and the sensitized group were 0.25 ± 0.07% and 0.24 ± 0.10%, respectively, with no significant difference ($P > 0.05$, Figure 1c).

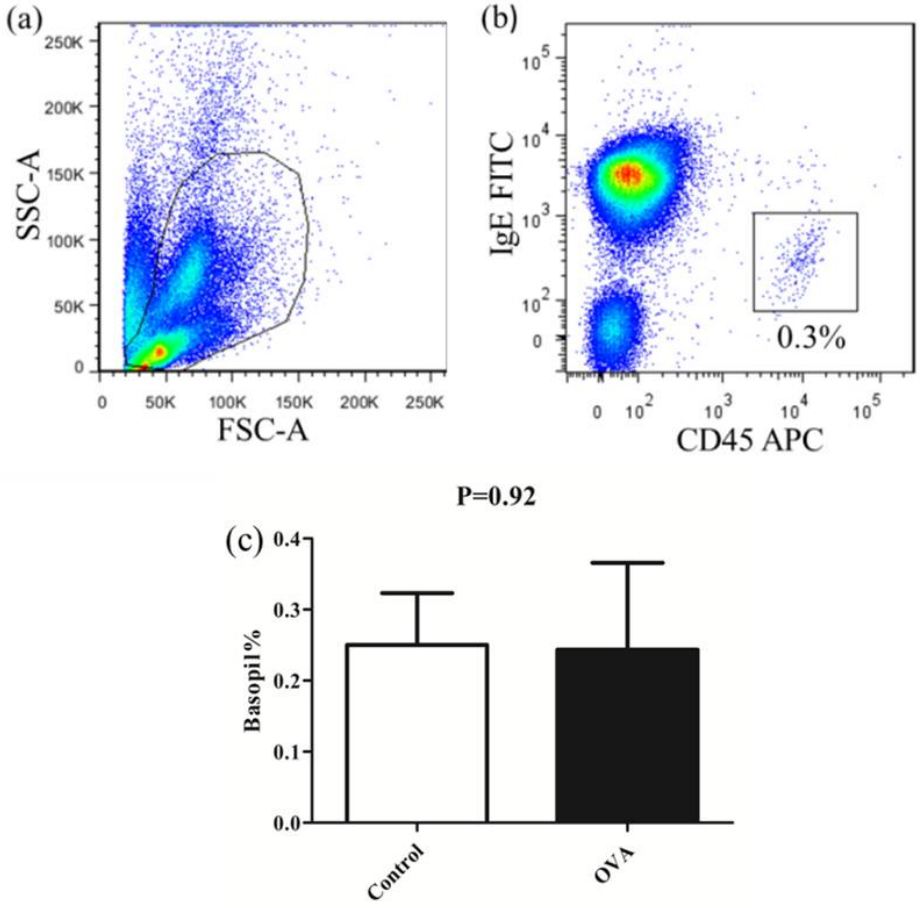

Figure 1. The gating strategy of basophils was determined by flow cytometry. (a) Original, the typical leukocyte region was gated on forward and side scatter (approximately 100 000 white cells). (b) Basophil populations were identified as CD45 APC^{low}/IgE $FITC^{high}$. (c) Automated basophil counting using flow cytometry between the control (NS) and OVA-sensitized groups, mean ± SD, n = 5, the OVA-sensitized group compared with the control group, $P > 0.05$.

3.1.2. Identification of rat basophils by blood smears

The rat peripheral blood smears were stained by Wright–Giemsa. The morphology of the rat basophils was examined, and dark purple black basophilic granules were observed in the cytoplasm, often covering the nucleus. The nucleus was mostly lobulated, which was not easily observed. There were no morphological changes between the control and OVA-sensitized groups (Figure 2a). The percentages of basophils in both groups were 0.3 ± 0.24% and 0.4 ± 0.37%, with no significant difference ($P > 0.05$) (Figure 2b).

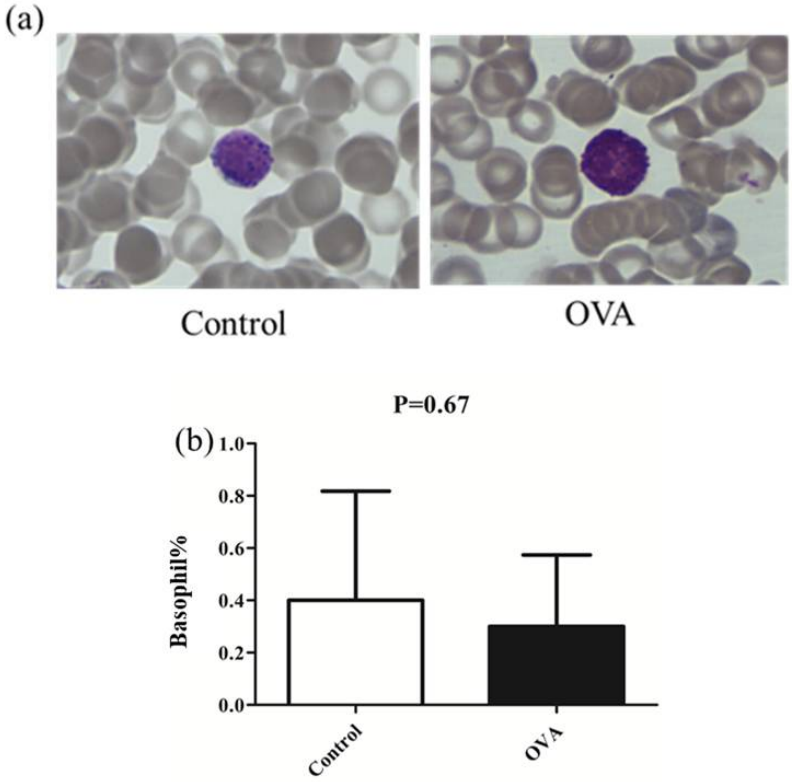

Figure 2. Blood smears identified basophils. Manual basophil counts in whole blood stained with Wright–Giemsa, × 1000. (a) The morphology of rat basophils and the numbers of dark purple black basophilic granules were not significantly different between the control (NS) and OVA-sensitized groups. (b) There was no significant difference in the number of basophils between the control and OVA-sensitized BN rats, mean ± SD, n = 5, the OVA-sensitized group compared with the control group, $P > 0.05$.

3.1.3. Results of two counting methods

Two counting methods yielded t-test P values of 0.36 ($P > 0.05$), indicating that CD45 APC^{low}/IgE $FITC^{high}$ can be used as markers to gate rat basophils.

3.2. Establishment of the basophil activation test

3.2.1. Passive cutaneous anaphylaxis

After verification with PCA, the rats were sensitized, OVA was combined with adjuvant to sensitize the rats, and the results of PCA were positive (Figure 3), suggesting that the rats produced the specific OVA-IgE.

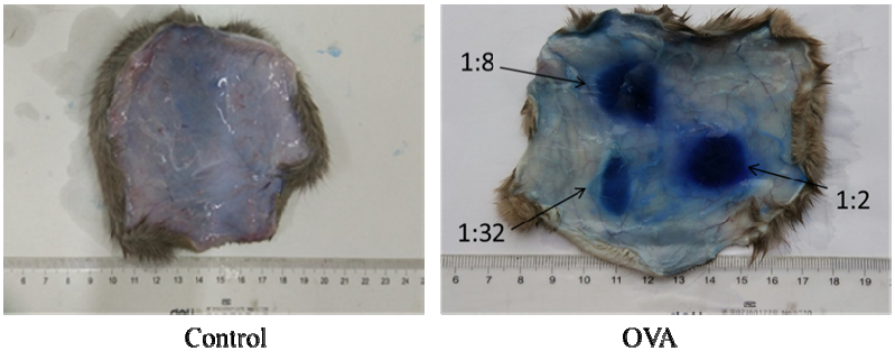

Figure 3. Passive cutaneous anaphylaxis (PCA) results of BN rats. n = 5, the control (NS) and OVA-sensitized rats were negative and positive (blue spot).

3.2.2. Basophil activation test

After verification of PCA, the basophils in the control group were incubated with PBS and OVA for 15 min in vitro, and the expression of CD63 on the surface of basophils was not induced, with percentages of 3.60 ± 2.82% and 3.51 ± 3.30% ($P > 0.05$), respectively.

The OVA-sensitized basophils were incubated with PBS and OVA for 15 min in vitro, and the expression of CD63 on the basophils was 1.78 ± 1.08% and 35.00 ± 23.90%, respectively. Compared with PBS, OVA significantly increased the expression of CD63 on basophils in vitro ($P < 0.05$ Figure 4), indicating that the BAT of rats was successfully built.

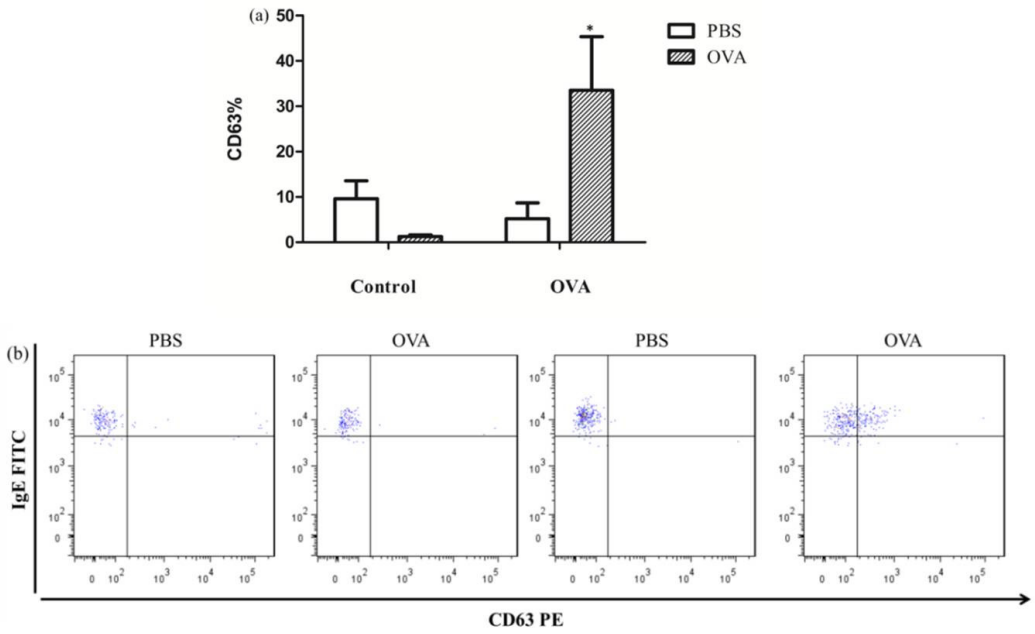

Figure 4. (a) Percentages of CD63 measured in the blood of BN rats sensitized with NS and OVA and incubated with the control (PBS) and OVA, respectively. Mean ± SD, n = 5, OVA compared with the control $^{*}P < 0.05$. (b) Flow diagram of Figure 4a: effect of CD63 expression in vitro.

3.3. Verification of rat basophil activation test

To verify that BAT can be used to detect the sensitization of LMWCs, we verified the effectiveness of BAT by using PG, which often causes allergic reactions in clinical practice.

3.3.1. Passive cutaneous anaphylaxis

In rats sensitized with PG/BSA and challenged with PG/OVA, the PCA showed negative results (Figure 5). For a high sensitivity of the BAT, the BAT was continued in vitro.

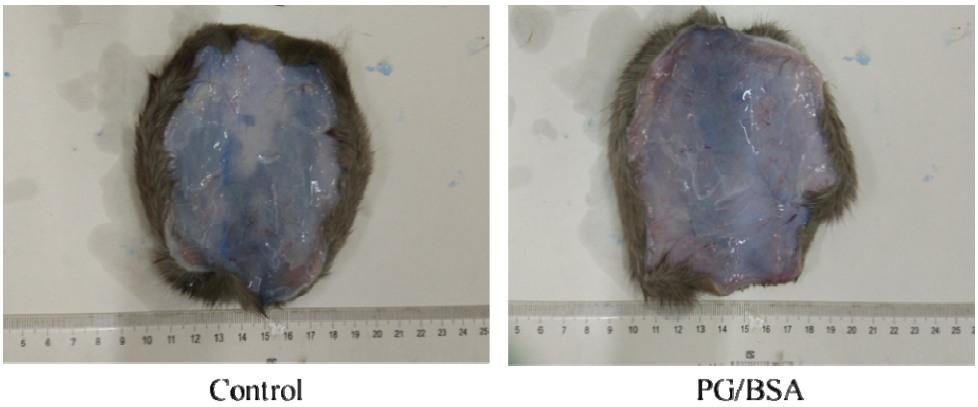

Figure 5. Passive cutaneous anaphylaxis results in BN rats. n = 5, control (NS) and PG/BSA-sensitized rats were negative and negative.

3.3.2. Basophil activation test

After the control group of rat basophils was incubated with PBS and PG/OVA for 15 min in vitro, the expression of CD63 in basophils was 4.7 ± 2.22% and 3.06 ± 1.18, respectively, with no significant difference ($P > 0.05$).

After the PG/BSA-sensitized rat basophils were incubated with PBS and PG/OVA for 15 min in vitro, the expression of CD63 on the basophils was 2.74 ± 2.10% and 31.10 ± 20.04%, respectively. Compared with PBS, PG/OVA significantly increased the expression of CD63 on the basophils in vitro ($P < 0.05$ Figure 6), suggesting that BAT has higher sensitivity than PCA.

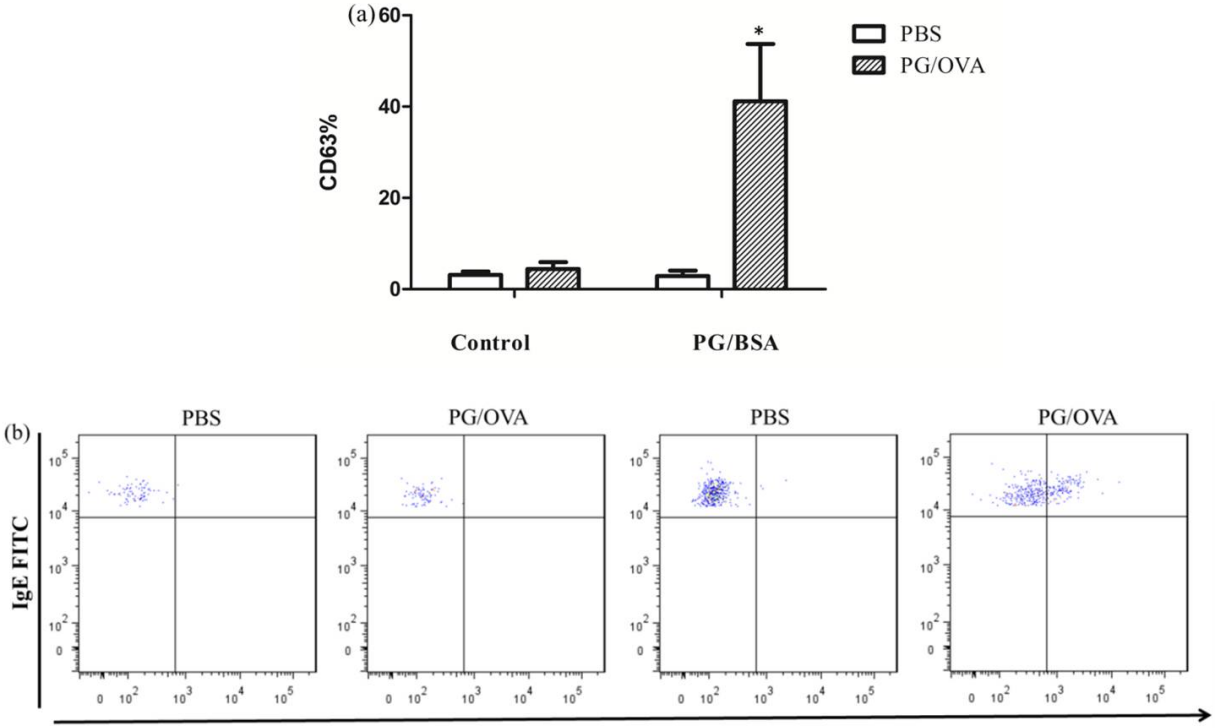

Figure 6. (a) Percentages of CD63 that were measured in the blood of BN rats sensitized with NS and incubated with control (PBS) or PG/OVA. Mean ± SD, n = 5, PG/OVA compared with the control $*P < 0.05$. (b) Flow diagram of Figure 6a: Effect of CD63 expression in vitro.

4. Discussion

BAT is widely used in the clinical diagnosis and research of allergic diseases [3,4], but little research has been conducted in animals. The first step in establishing a rat BAT model is to determine the basophil population in whole blood using classic blood smear methods and flow cytometry. CD45 APC^{low}/IgE $FITC^{high}$ were used as markers for the identification of rat basophils based on the clinically common BAT protocol in flow cytometry for CD45/IgE [7]. The results showed that CD45 APC^{low}/IgE $FITC^{high}$ could be used to obtain rat basophils.

Since the blood smear count is a clinically reliable cell counting method [8], this technique was used in this project to observe the morphology of basophilic granulocytes and determine the count in rats. A significant increase in the number of basophils in the peripheral blood of allergic patients has been confirmed, but whether basophils show changes before and after sensitization has not been studied. Flow cytometry and blood smears have been used for research in rats in this project. The results showed that there was no significant difference between the control and sensitized groups in morphology and quantity. There was also no significant difference between the two counting methods, consistent with previous studies [9], indicating that CD45 APC^{low}/IgE $FITC^{high}$ can be used as markers to gate rat basophils.

CD63 has been widely used as a marker of human basophil activation in clinical BAT [10–12], but studies on rats have not been performed. Therefore, the rats were sensitized with OVA, and the

peripheral blood was incubated with OVA in vitro to observe the expression of CD63. First, PCA was used to verify whether the rat was sensitized. The results of the control group were negative for PCA, and CD63 expression in basophils was not stimulated by PBS or OVA in vitro. The positive results of PCA in the OVA-sensitized rats indicated that OVA-IgE was produced in rats, basophils were activated after OVA incubation in vitro, and CD63 expression was significantly increased, indicating that the expression of CD63 was dependent on basophils activated by the antigen-specific IgE pathway. The BAT of rats was successfully constructed, providing an experimental basis for evaluating the sensitization of LMWCs.

PCA is a preclinical method for evaluating drug sensitization, and various experimental factors may affect the occurrence of allergic reactions, thereby affecting the blue spot size. For example, the size of the PCA is affected by the serum IgE content [13]. BAT relies on cell surface-specific IgE binding and subsequent aggregation of the receptor (FcεRI) to activate the expression of markers. There is no strict requirement on the size of receptor aggregation [14,15].

The BAT of rats was verified by PG and the PG/BSA-sensitized rat, and the blood was challenged by PG/OVA in vitro, consistent with the mechanism of LMWC sensitization: the mechanism of type I hypersensitivity induced by the hapten-carrier complexes [16]. In this project, we verified by PCA that basophils in the PG/BSA-sensitized rats were sensitized or that the rats produced specific PG-IgE. The PCA results were negative but could not explain why there was no IgE production in rats. However, CD63 was significantly increased after the basophils were incubated with PG/OVA in vitro. The reason may be that the amounts of PG-IgE or PG/BSA were lower with poor IgE aggregates.

Sometimes, skin tests of patients who are allergic to PG are negative. In contrast, the sensitization of PG can be detected with high sensitivity in BAT [17]. The sensitization of LMWCs and macromolecular proteins in rats provided new ideas for the preclinical evaluation of the sensitization of LMWCs.

However, this project has drawbacks, and BAT cannot distinguish whether activated basophils are mediated by the IgE pathway or other mechanisms [18]. Not all CD63 is upregulated in the activation of basophils, and approximately 5% to 10% of individuals fail to increase CD63 in IgE-mediated basophil activation pathways, causing false negative results in the BAT [19]. The above issue will be noteworthy for BAT in research and application.

5. Conclusion

This project successfully identified rat basophils and successfully established a BAT, which can detect sensitization of PG, suggesting rat BAT can be used to evaluate the potential sensitization of new drugs.

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role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interests

All authors declare no conflicts of interest in this paper.

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