

The attenuative effect of purified protein derivative sensitization on T helper 2 reaction and eosinophil infiltration of the lung in ovalbumin sensitized mice

Cui Xinle 崔新乐, Guo Zijian 郭子健, Xu Wenbing 许文兵, Chen Yong 陈勇 and Zhu Yuanjue 朱元珩

Objectives To identify the effects of tuberculin purified protein derivative (PPD) sensitization on attenuating pulmonary T helper 2 (Th2) reaction and eosinophil infiltration in ovalbumin sensitized mice, and to search for the possibility of its clinical use in the management of asthma in a new way.

Methods Sixty C57BL/6 mice were sensitized with PPD and then with ovalbumin and aluminum hydroxide, and randomized into 4 groups: ovalbumin (OVA), pre-PPD, post-PPD and control groups. Aerosol PPD were administered 3 h before or after ovalbumin challenge in the pre-PPD and post-PPD groups respectively, and control group received aerosol PPD only. IL-4, IL-5 expression was detected by immunocytochemistry in situ hybridization. Lung slides were stained with eosin and hematoxylin, and pathological changes were observed.

Results Ovalbumin aerosol inhalation caused a mixed inflammatory infiltration dominated by CD4⁺ T lymphocytes and eosinophils in the lung of sensitized mice. 87.5% – 89.7% and 89.0% – 89.2% of the CD4⁺ T lymphocytes were IL-4 mRNA⁺ and IL-5 mRNA⁺ respectively. 88.7% – 91.2% of IL-4 mRNA⁺ cells and 89.8% – 90.6% of IL-5 mRNA⁺ cells were CD4⁺ T lymphocytes in OVA group. Aerosol administration of PPD markedly suppressed IL-4 and IL-5 expression, and lung eosinophil infiltration. It was more effective in pre-PPD group. 76.6% – 78.0% of IL-4 mRNA⁺ and 73.8% – 79.7% of IL-5 mRNA⁺ cells were CD4⁺ and 78.1% – 84.9% and 78.4% – 85.3% of the CD4⁺ cells were IL-4 mRNA⁺ or IL-5 mRNA⁺ respectively in pre-PPD group, both were markedly lower than that of OVA group. CD4⁺ percentage of IL-4 mRNA⁺ and IL-5 mRNA⁺ cells were 80.7% – 82.0% and 78.0% – 83.9% in post-PPD group, which were markedly lower than that of OVA group.

Conclusions Sensitization with PPD by intraperitoneal injection and then challenged by PPD inhalation markedly suppressed IL-4, IL-5 expression and

eosinophil infiltration, and attenuated pulmonary Th2 reaction in ovalbumin sensitized mice. This induces Th1 type reaction and inhibits Th2 cell differentiation. It may be beneficial for glucocorticoids dependent or resistant patients.

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Asthma is a chronic inflammatory condition of the airways. Histological examination of allergic asthmatic airways reveals epithelial damage, microvascular leakage, mucus hypersecretion, deposition of collagen beneath the membrane and a mixed cellular infiltrate dominated by eosinophils and T lymphocytes, and a similar pathological change was found in occupational and late onset "intrinsic" asthmatic airways. A considerable amount of evidence of both animal experiments and clinical studies suggests that T lymphocytes serve to modulate the inflammatory response and that Th2 cell type cytokine interleukin (IL)-4 and IL-5 produced mainly by CD4⁺ T lymphocytes. These may play a key role in the pathogenesis of asthmatic airway inflammation.^{1,2} Airway inflammation leads to hyperresponsiveness of the bronchi which is characterised by bronchial irritability, fluctuating peak expiratory flow measurements, and a tendency to bronchoconstrict on exertion or when exposed to non-specific respiratory irritants. Th1 reaction cytokine interferon (INF)- γ inhibits Th2 cell differentiation. Intraperitoneal infusion of INF- γ attenuated CD4⁺ T lymphocyte and eosinophil infiltration in trachea and aerosol administration of INF- γ decreased airway responsiveness in ovalbumin sensitized mice. We hypothesized that Th1 reaction might inhibit Th2 reaction through INF- γ production.

Department of Respiratory Diseases, PUMC Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China (Cui XL, Guo ZJ, Xu WB, Chen Y and Zhu YJ)

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In our experiment, aerosol tuberculin purified protein derivative (PPD) were administered to ovalbumin and PPD sensitized C57BL/6 mice to identify its effects on pulmonary Th2 reaction and eosinophil infiltration, the mechanism of which may be helpful in the management of asthma.

METHODS

Mice sensitization and challenge

Specific pathogen-free C57BL/6 mice, 6–8 weeks old, were supplied by Animal Center of CAMS and PUMC. Sixty mice were immunized intraperitoneally twice with 0.5 μ g PPD at a 2-week interval, and then immunized with OVA by intraperitoneal injection of 10 μ g ovalbumin (Sigma Chemical Co, St Louis Mo) in 4 mg aluminum hydroxide on the 17th and 24th day of the experiment. Two weeks after the last immunization, mice were randomized into 4 groups: OVA, pre-PPD, post-PPD and control. Mice of the OVA, pre-PPD and post-PPD group were exposed to aerosolized ovalbumin (0.5%) in a sealed chamber for 30 minutes. The aerosolized ovalbumin or PPD was produced by an ultrasonic nebulizer at a flow rate of 4 L/min. Pre-PPD group was administered with aerosol PPD (0.375 μ g/ml) for 30 minutes at 3 h before ovalbumin challenge. Post-PPD group was administered with aerosol PPD at 3 h after ovalbumin challenge. Twelve hours later, mice were re-challenged with aerosolized ovalbumin and/or PPD as stated above. At 6 h, 12 h and 32 h after the secondary challenge with ovalbumin, mice were killed by cervical dislocation. 0.4 ml 4% paraformaldehyde-PBS was instilled through trachea. The lungs were removed and fixed in 4% paraformaldehyde-PBS for 3 h. After being washed twice in 15% sucrose-PBS, the mice lungs were snap-frozen in isopentane, cooled in liquid nitrogen and stored at -70°C .

Preparation of probes

E. coli HB 101 strain was transformed with plasmid PGH-IL-4 and PGH-IL-5 (a gift from Dr. Sunying, National Heart and Lung Institute, UK), containing human IL-4 and IL-5 cDNA respectively, and incubated in LB medium. Plasmids were extracted by alkali lysis method and purified by precipitation with polyethylene glycol. PGH-IL-4 was linearized with Sph I (antisense) or EcoRI (sense), PGH-IL-5 was linearized with Xba I (antisense) or Bam HI (sense). Linearized templates were labeled with digoxigenin-11-UTP by *in vitro* transcription using RNA Dig-labeling kit (Boehringer Mannheim, Mannheim Germany), according to standard protocol from the manufacturer. Labeled probe produced was 8–10 μ g per μ g template measured by 1% agarose electrophoresis. The specificity of labeled RNA probe was confirmed by using a dot-blot detection system (Boehringer Mannheim).

Immunohistochemical staining

10 μ m cryostat sections were freshly cut from mouse lung, mounted on 0.1% poly-lysine-coated-slides (RNase free) and air dried overnight at 37°C . Immunohistochemistry was performed using the streptavidin-biotin-peroxidase method. Briefly, slides were immersed in 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4) for 5 minutes, then incubated with rat anti-mouse CD4 or CD8 monoclonal antibody for 1 hour. Slides were then incubated with a biotin-labeled goat anti-rat antibody (Dako) and streptavidin, each for 30 minutes. Goat anti-rat IgG and streptavidin-AP were diluted in PBS with 3% BSA. In order to avoid loss of immunochemical signals during the *in situ* hybridization procedure, signals were enhanced by repeating steps 2 and 3, 10 minutes for each. Washing were performed between all steps (3×2 min) with PBS. To develop the reaction, fast red (Sigma) was used as the chromogen and the intensity of immunostaining reaction was adjusted by light microscopy. In order to avoid RNase contamination, all solutions used were made up in 0.1% diethylpyrocarbonate (DEPC) treated distilled water.

In situ hybridization

The method was applied according to Ying et al,³ and some modifications were made. After immunochemical staining, the same slides were immediately immersed in 4% paraformaldehyde/PBS for 5 minutes, then treated with 0.3% Triton-X 100 in PBS for 10 min. After a brief wash in DEPC treated PBS, sections were exposed to proteinase K solution (1 μ g/ml in 20 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 7.2) for 15 minutes at 37°C . After a brief rinse in PBS, slides were immersed in 4% paraformaldehyde in PBS for 5 minutes, transferred to DEPC treated PBS, and then air-dried. Negative controls were slides treated with 100 μ g/ml of RNase (Sigma) at 37°C for 30 minutes to digest all tissue mRNA, including IL-4 and IL-5.

Concentrations of digoxigenin-labeled IL-4 and IL-5 probes, both sense and antisense, were adjusted to 5 μ g/ml–10 μ g/ml in hybridization buffer that consisted of 50% formamide, 5 $\times$ Denhardt's solution, 5 $\times$ standard saline citrate buffer (SSC), and 300 μ g/ml denatured salmon sperm DNA (Sigma). A volume of 30 μ l of the hybridization mixture was added to each section. The sections were covered and incubated overnight at 40°C in a humid chamber. The slides were then washed sequentially in 4 $\times$ SSC at 40°C for 10 minutes twice, incubated with 20 μ g/ml RNase A in 2 $\times$ SSC for 30 minutes at 37°C to remove any unhybridized RNA, and washed in 2 $\times$ SSC at room temperature. After a brief wash in TBS, sections were incubated with 3% bovine serum albumin/TBS for 10 minutes to reduce nonspecific background, then incubated with 1:500 dilutions of Dig-AP conjugate (sheep polyclonal anti-digoxigenin antibody Fab

fragment) in TBS containing 0.1% Triton-X 100 in a humid box for 10 h at room temperature. The slides were washed 3 times in TBS, each for 15 minutes, followed by incubation with equalization buffer (0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, 50 mm MgCl₂, pH 9.5) for 5 minutes. Color development was achieved by adding a fresh prepared substrate solution consisting of 0.2 mg X-phosphate-5-bromo-4-chloro-3-indol phosphate (BCIP) and 0.4 mg nitroblue tetrazolium (NBT) salt per ml of equalization buffer to the slides for 20 minutes at room temperature. The slides were transferred to TBS and washed in tap water, counterstained with hematoxylin for 5 seconds, and mounted with glycerol gelatin.

The following control experiments were also performed: (1) running in situ hybridization (ISH) experiments without previous immunocytochemistry (ICC); (2) ICC alone; (3) using sense digoxigenin-labeled riboprobe; (4) pretreatment of slides with RNase A before ISH.

The slides were counted field by field under a light microscope for cells positive for ICC, ISH, and double ICC and ISH, at least two sections and a median of 15 field were counted. The results were expressed as number of positive cells per high power field and as percentage of IL-4 mRNA⁺

and IL-5 mRNA⁺ cells that coexpressed immunoreactivity for CD4 or CD8.

Hematoxylin and eosin staining

The slides were stained with hematoxylin and eosin. Eosinophils were counted field by field as mentioned above, and expressed as number of eosinophils per high power field.

RESULTS

Effects of IL-4 expression after ovalbumin challenge and PPD inhalation

IL-4 mRNA⁺ cells of lung tissues were 85.9%, 57.1% and 99.5% per high power field (400×) slides in OVA group at 6 h, 12 h and 32 h after the secondary challenge with ovalbumin respectively. No positive cell was found in the lung slides of control group. IL-4 expression was markedly suppressed by PPD inhalation, especially in the pre-PPD group. IL-4 mRNA⁺ cells in pre-PPD and post-PPD group were 16.5%, 12.4%, 34.6% and 52.0%, 36.1%, 64.1% in the lung slides at 6 h, 12 h, 32 h after the secondary ovalbumin challenge per 400× field respectively. There were significant differences between OVA and PPD groups (Table 1).

Table 1. IL-4 mRNA⁺ cells and the ratio of IL-4⁺ to CD4⁺ cells ($\bar{x} \pm s$)

Groups	IL-4 ⁺ (%)			CD4 ⁺ /IL-4 ⁺ (%)			IL-4 ⁺ /CD4 ⁺ (%)		
	6 h	12 h	32 h	6 h	12 h	32 h	6 h	12 h	32 h
Control	0	0	0						
OVA	85.9 ± 10.2	57.1 ± 9.5	99.5 ± 6.0	89.7 ± 1.0	87.5 ± 2.7	88.0 ± 1.6	90.2 ± 1.8	88.7 ± 2.5	91.5 ± 0.9
Pre-PPD	16.5 ± 5.2 [*]	12.4 ± 3.9 [*]	34.6 ± 13.8 [*]	76.6 ± 2.2 [*]	76.9 ± 2.4 [*]	78.0 ± 1.9 [*]	84.9 ± 1.1 [#]	78.1 ± 2.7 [*]	83.2 ± 3.2 [*]
Post-PPD	52.0 ± 6.0 [*]	36.1 ± 7.7 [*]	64.1 ± 9.9 [*]	80.7 ± 0.6 [#]	82.1 ± 4.1 [#]	81.3 ± 6.6 [#]	87.3 ± 1.5 [#]	88.0 ± 3.1	88.9 ± 1.3

* $P < 0.01$, compared with OVA group; + $P < 0.01$, compared with post-PPD group; # $P < 0.05$, compared with OVA group.

Effects of IL-5 expression after ovalbumin challenge and PPD inhalation

IL-5 mRNA⁺ cells of lung tissues were 79.2%, 63.5% and 74.6% per high power field (400×) slides in OVA group at 6 h, 12 h and 32 h after the secondary challenge with ovalbumin. IL-5 expression was suppressed by PPD inhalation as well as IL-4. IL-5 mRNA⁺ cells in

pre-PPD and post-PPD group were 5.3%, 21.8%, 33.5% and 32.3%, 34.6%, 39.6% in the lung slides at 6 h, 12 h, 32 h after the secondary ovalbumin challenge per 400× field respectively. IL-4 mRNA⁺ and IL-5 mRNA⁺ cells were reduced sharply in the lung slides of pre-PPD and post-PPD groups than those of OVA group. There were significant differences between the two PPD groups (Table 2).

Table 2. IL-5 mRNA⁺ cells and the ratio of IL-5⁺ to CD4⁺ cells ($\bar{x} \pm s$)

Groups	IL-5 ⁺ (%)			CD4 ⁺ /IL-5 ⁺ (%)			IL-5 ⁺ /CD4 ⁺ (%)		
	6 h	12 h	32 h	6 h	12 h	32 h	6 h	12 h	32 h
Control	0	0	0						
OVA	79.2 ± 4.2	63.5 ± 4.7	74.6 ± 7.8	89.2 ± 1.1	89.0 ± 0.9	89.2 ± 1.6	89.7 ± 1.3	90.4 ± 1.6	90.6 ± 1.2
Pre-PPD	5.3 ± 2.4 [*]	21.8 ± 5.1 [*]	33.5 ± 7.5 [*]	73.8 ± 6.8 [*]	79.2 ± 1.7 [*]	79.7 ± 2.0 [*]	78.4 ± 7.3 [*]	85.0 ± 3.1 [#]	85.3 ± 3.1 [#]
Post-PPD	32.3 ± 6.4 [*]	34.6 ± 2.2 [*]	39.6 ± 6.7 [*]	78.4 ± 2.1 [*]	78.0 ± 5.1 [*]	83.9 ± 5.8 [*]	85.3 ± 4.4	88.4 ± 4.8	88.0 ± 4.7

* $P < 0.01$, compared with OVA group. + $P < 0.01$, compared with post-PPD group. # $P < 0.05$, compared with OVA group.

Difference of CD4⁺ and CD8⁺ T lymphocytes in cytokine expression

87.5% - 89.7% of the IL-4 mRNA⁺ cells were

CD4⁺, and 89.0% - 89.2% of IL-5 mRNA⁺ cells were CD4⁺ in OVA group. 88.7% - 91.2% of CD4⁺ cells expressed IL-4, and 89.7% - 90.6% of CD4⁺ cells

expressed IL-5 in OVA group. CD4⁺ percentages of IL-4 mRNA⁺ and IL-5 mRNA⁺ cells were 76.6% - 78.0% and 73.8% - 79.7% respectively in pre-PPD group, significantly lower than those of OVA group. 78.1% - 84.9% and 78.4% - 85.3% of CD4⁺ cells expressed IL-4 or IL-5 respectively in pre-PPD group, also significantly lower than that of OVA group. CD4⁺ percentages of IL-4 mRNA⁺ and IL-5 mRNA⁺ cells were 80.7% - 82.0% and 78.0% - 83.9% respectively in post-PPD group, significantly lower than those of OVA group, but percentages of IL-4, IL-5 expression of CD4⁺ cells only in a few subgroups were lower than that of OVA group. Only few CD8⁺ cells expressed IL-4 or IL-5, less than 2% (Tables 1 and 2).

Effects of dexamethasone and methotrexate on eosinophil infiltration

The lung slides of OVA group contained 26.5 ± 2.5 , 35.0 ± 1.2 and 26.8 ± 3.8 ($\bar{x} \pm s$, the same with below) eosinophils per 400 $\times$ field respectively at 6 h, 12 h, 32 h after the secondary ovalbumin challenge. The lung slides of both pre-PPD and post-PPD groups contained much less eosinophils than those of OVA group. Eosinophils were 4.0 ± 1.6 , 13.6 ± 1.6 , 9.5 ± 3.0 in pre-PPD group ($P < 0.01$) and 2.9 ± 1.5 , 14.1 ± 1.2 , 9.4 ± 0.8 in post-PPD group ($P < 0.01$) per 400 $\times$ field respectively at 6 h, 12 h and 32 h after the secondary ovalbumin challenge (Figs. 1 and 2).

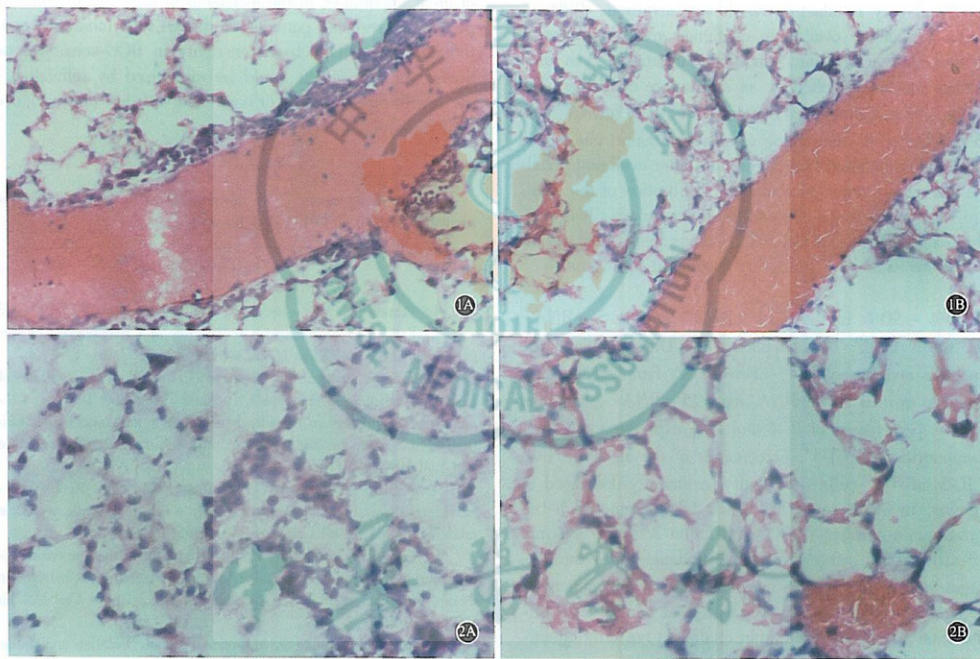


Fig. 1. Rat lung tissues at 12 h after the secondary ovalbumin challenge (original magnification $\times 400$). A: OVA group: white blood cells emigrated in venule, massive exudation occurred, more eosinophils infiltrated in peri-venule tissue; B: Post-PPD group: few white blood cells emigrated in venule, no exudation, a few eosinophils infiltrated.

Fig. 2. Rat lung tissues at 32 h after the secondary ovalbumin challenge ($\times 400$). A: OVA group: massive edema of mesenchyma, large amount of eosinophils and lymphocytes infiltrated; B: Pre-PPD group: capillaries were enlarged, with a few eosinophils infiltrated.

DISCUSSION

Asthma is a disease of chronic airway inflammation which presents with a mixed cell infiltration dominated by T lymphocytes and eosinophils beneath the bronchial epithelium. IL-4 and IL-5 produced mainly by CD4⁺ Th2 cells play a key role in orchestrating inflammatory response. IL-4 acts

directly on the precursor T cells to induce CD4⁺ Th2 cell differentiation, and Th2 cells release more IL-4, and more CD4⁺ Th2 cells differentiated, a positive feedback that leads to Th2 cell infiltration in the airways, which plays a critical role in asthmatic pathogenesis.⁴ In addition, IL-4 stimulates and switches the immunoglobulin production of B cells to IgE. As CD4⁺ Th2 cells play a critical role in

orchestration of airway inflammation, decrease in the number of IL-4⁺ or IL-5⁺ T lymphocytes should serve as the criteria of asthma treatment.⁵ Th1 cells produce INF- γ , TNF- β , etc., which is the cause of classical delayed type hypersensitivity. PPD induces Th1 cell differentiation *in vitro*, and Th1 cytokine INF- γ prevents Th2 cell differentiation from Th0 cells. If a Th2 reaction could be induced in the airways, asthmatic airway inflammation would be markedly suppressed.

Human IL-4 and IL-5 have over 80% nucleotide sequence homologous to that of murine.^{6,7} We did *in situ* hybridization using human IL-4 and IL-5 RNA probe in the detection of murine IL-4 and IL-5 mRNA under proper condition, and very satisfactory result was acquired. PPD inhalation 3 h before ovalbumin challenge greatly suppressed IL-4 and IL-5 expression, and PPD inhalation 3 h after challenge was not as effective as 3 h before challenge, but the lung slides of this group contained less IL-4 mRNA⁺ and IL-5 mRNA⁺ cells than those of only ovalbumin challenged group. PPD inhalation both before and after ovalbumin challenge markedly attenuated eosinophil infiltration of the lung. There was no difference between these two groups. It is likely that except for IL-5, Th1 cells secrete some other mediators that are capable of inhibiting eosinophil infiltration.

80% - 90% of the IL-4 mRNA⁺ and IL-5 mRNA⁺ cells were CD4⁺ T lymphocytes. Others might be eosinophils and basophils.³ Few CD8⁺ lymphocytes were detected to express IL-4 mRNA⁺ or IL-5 mRNA⁺, less than 2%. The percentages of IL-4 and IL-5 expression of CD4⁺ T lymphocytes and CD4⁺ percentages of the IL-4 mRNA⁺ and IL-5 mRNA⁺ cells were both significantly decreased in the group, in which aerosol PPD was administrated 3 h before ovalbumin challenge. PPD inhalation 3 h later was not as effective as 3 h before ovalbumin challenge, only the percentages of IL-4 and IL-5 expression of CD4⁺ T lymphocytes were significantly decreased. It seems that aerosol PPD administration 3 h before ovalbumin challenge not only attenuates CD4⁺ T lymphocytes infiltration of the lung, but also suppresses IL-4 and IL-5 expression of CD4⁺ lymphocytes, which is the symbol of Th2 cell differentiation. On the contrary, in the group with PPD administration 3h after ovalbumin challenge, only CD4⁺ lymphocyte infiltration was attenuated, but the percentages of IL-4 and IL-5 expression of CD4⁺ lymphocytes were not markedly decreased.

"De-sensitization method" has been proved ineffective in the treatment of asthma, though glucocorticoids are considered the most effective drug. But glucocorticoids need a longer period to show its full effects, and is less effective in anti-inflammation. We discovered in our previous experiments that IL-5 expression was suppressed 12 h after dexamethasone inhalation, IL-4 expression and eosinophil infiltration were attenuated 24 h after dexamethasone inhalation. If asthmatic patients could be sensitized with BCG inoculation, and then challenged with aerosol PPD to induce Th1 type reaction in the airways, the Th2 type reaction would be greatly suppressed. Pre-inhalation of PPD could prevent Th2 cell differentiation from Th0 cells, and then prevent asthmatic exacerbation without side effects of glucocorticoids, which is of great importance in the management of glucocorticoids dependent or resistant patients. Although PPD inhalation could induce delayed type hypersensitivity in BCG-sensitized patients, its side effects could be minimized by inhalation of the lowest dose of PPD, or combined with low dose of glucocorticoids inhalation.

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