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Cimetidine inhibits production of interferon γ and tumor necrosis factor α by splenocytes in aplastic anemic mice

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KEY WORDS cimetidine; aplastic anemia; tumor necrosis factor; interferon type Ⅱ; enzyme-linked immunosorbent assay

ABSTRACT

AIM: To study the effects of cimetidine (Cim) on the production of interferon γ (IFN γ) and tumor necrosis factor α (TNF α) by splenocytes in immune-derived aplastic anemic (AA) mice. **METHODS**: Aplastic anemic mice model was constructed first, and then the splenocytes were induced to secrete IFN γ and TNF α. Concentration of IFN γ was assayed using sandwich ELISA, while that of TNF α was measured with L929 cytotoxicity methods. **RESULTS**: (1) Concentrations of IFN γ and TNF α secreted by splenocytes from AA mice were (137 ± 36) ng/L and (6 ± 3) μg/L, respectively, much more than the irradiated and the control mice. (2) Treatment with Cim 10 μmol/L reduced the concentrations of IFN γ and TNF α to (14 ± 8) ng/L and (2.7 ± 0.6) μg/L, respectively. **CONCLUSION**: Cim could effectively reduce the production of IFN γ and TNF α from splenocytes of AA mice.

INTRODUCTION

Many studies have shown that aplastic anemia (AA) is a kind of disease with disturbed immunity status[1,2]: abnormal $CD4^+/CD8^+$ lymphocytes ratio and high concentration of hematopoietic suppressive factors produced by T lymphocytes. Fundamentally, abnormally activated T lymphocytes played an important role in the formation and development of AA. Cimetidine, a specific histamine type Ⅱ receptor (H_2R) antagonist, traditionally used as a therapeutic agent for ulcer, is found to be involved in modulating the function of $CD8^+$ T lymphocytes. It could decrease the number of H_2R, and could reverse the inhibition of IgM and IgG synthesis by $CD8^+$ lymphocytes[3]. Recently, it was found that Cim could partially reverse the hematopoietic suppressive effects by $CD8^+$ T lymphocytes in AA patients[4]. These findings are inspiring since AA is a kind of refractory disease, with its therapy to date having no satisfactory results. Using the AA mice model, we investigated the mechanism underlying the action of Cim on the lymphocytes.

MATERIALS AND METHODS

Animals Balb/c mice (8-12 weeks, ♀ + ♂, Grade Ⅱ), DBA/2 mice (6-14 weeks, ♀ + ♂, Grade Ⅱ), were obtained from Beijing Experimental Animal Center.

Cell lines and reagents Murine fibroblast L929 cells were maintained in our lab and cultured to confluency at 37 ℃ in a humidified atmosphere of 5 % CO_2 and 95 % air in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10 % new-born bovine serum (NBS). IMDM medium, Cim, LPS, and actinomycin D (ActD) were purchased from Sigma Co (USA), and phytohemagglutinin P (PHA-P) was obtained from Serva (Germany), rh TNFα was a product of R&D (USA). NBS was purchased from Sijiqing Bioengineering Co (Hangzhou). Mouse IFN γ EIA kit (Pharmingen, USA) was a gift from Prof YI Xin-Yuan (Department of Parasitology, Hunan Medical University).

Construction of AA model According to Yao's method[5], immune-induced AA mice were prepared. In brief, the lymph nodes and the thymus of donor DBA/2 mice were obtained aseptically, then the single cells suspension was prepared using homogenizer, trypan blue exclusion tests showed that more than 95 % of the cells were alive. The recipient Balb/c mice were given 6 Gy total body irradiation using ^{60}Co sources, immediately after the irradiation, total of 1×10^6 mixed cells (2 lymphocytes: 1 thymocyte) were transfused via tail vein. Then

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the recipients were fed with care under normal conditions. In the meantime, the irradiated group which was given only 6 Gy irradiation without transfusion and the control group were set up.

Preparation of splenocytes The model mice were sacrificed using cervical dislocation 12 – 14 d after the transfusion. The spleens were taken out aseptically, and the splenocytes were crushed out using forceps, then passed through 23 gauge syringe several times to get single cell suspension. Trypan blue exclusion tests showed that 90 % cells were alive. Then the cells were adjusted to appropriate concentration. Mice of irradiated and control groups were treated similarly.

Assay for IFN γ produced by splenocytes induced with PHA One million splenocytes cultured in IMDM containing PHA-P 50 mg/L, 20 % NBS was incubated in 5 % CO_2, 37 ℃, and 100 % humidity for 72 h. In the experimental group, Cim 10 $\mu mol \cdot L^{-1}$ was added into the culture. After centrifugation and filtration using 0.4-μm millipore membrane, and the culture supernatant was obtained. The concentration of IFN γ was determined using sandwich ELISA according to the manual. Briefly, ninety six-well microtiter plates were coated with capture antibody overnight at room temperature. Then the plates were blocked with 3 % non-fat milk for 1 h at 37 ℃. After washing, each well was added 50 μL detecting antibody and equal volume of collected supernatant or diluted standard IFN γ provided with the Kit or IMDM as a control, and the plates were then incubated at room temperature for 2 h. The plates were washed 5 times before 100 μL of substrate solution was added into each well, then the plates were incubated at room temperature for 30 min in the dark. The absorbance at 570 nm was measured with a Model ∑960 microplate reader (Japan).

Assay for TNF α produced by splenocytes induced with LPS One million splenocytes cultured in IMDM containing LPS 1.01 mg/L was incubated in 5 % CO_2, 37 ℃, and 100 % humidity for 12 h. In the experimental group, Cim 10 μmol/L was added into the culture. After centrifugation and filtration through 0.4-μm millipore membrane, the culture supernatant was obtained. The TNF α activity was assessed using cytotoxicity method. Twenty thousand L929 cells in the exponential stage were plated in 96-well microtiter plate, incubated in 5 % CO_2, 37 ℃ for 24 h to form a subconfluent layer, then the supernatant was aspirated, supplemented with 100 μL sample or serially diluted rh TNF α or IMDM per well. ActD 1 mg/L was also added. After 12 h of incubation at 37 ℃, the supernatant was aspirated. After the plates were washed with PBS, 100 μL of 0.25 % crystal violet was added into each well for 10 min at room temperature. The plates were washed with PBS thrice, then citric sodium buffer was used to dissolve the remaining stain. The absorbance at 570 nm was measured with a Model ∑960 microplate reader.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and the data for multiple comparisons was performed by ANOVA followed by Dunnett's test.

RESULTS

Effects of Cim on IFN γ production The concentration of IFN γ was determined using the standard curve. Results showed that the splenocytes from AA model mice produced much more IFN γ (137 ± 36) ng/L than the irradiated mice (12 ± 7) ng/L and control mice (8 ± 8) ng/L after induction with PHA-P ($P < 0.01$). When Cim 10 μmol/L was added to the culture, IFN γ secreted by AA splenocytes reduced to (14 ± 8) ng/L ($P < 0.01$). While in the irradiated and the control mice it was (9 ± 8) ng/L and (16 ± 12) ng/L, respectively. There was no significant difference after Cim treatment ($P > 0.05$). (Fig 1)

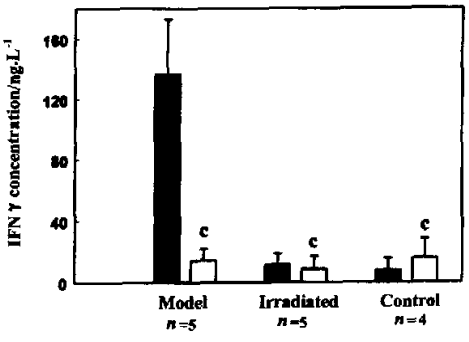

Fig 1. The effects of cimetidine on the production of IFN γ from spleen lymphocytes induced by PHA-P. ■: without treatment of Cim; □: treated with Cim 10 μmol/L. °$P < 0.01$ compared with untreated model mice.

Effects of Cim on TNF α production The concentration of TNF α was calculated using the standard curve. TNF α produced by AA mice splenocytes (6 ± 3) μg/L was more than that of irradiated mice (2.8 ± 0.7) μg/L and control mice (2.48 ± 0.13) μg/L ($P <$

0.05). After treatment with Cim 10 μmol/L, the TNF α concentration in AA mice decreased from (6 ± 3) μg/L to (2.7 ± 0.6) μg/L ($P < 0.05$). But there was no significant difference in irradiated (3.1 ± 0.6) μg/L) and control mice (2.63 ± 0.22) μg/L after Cim treatment (Fig 2).

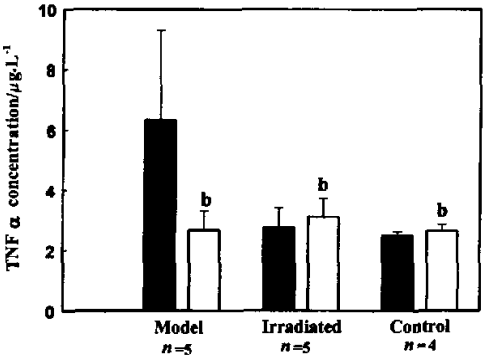

Fig 2. The effects of cimetidine on the production of TNF α from spleen lymphocytes induced by LPS. ■: without treatment of Cim; □: treated with Cim 10 μmol/L. $^bP < 0.05$ compared with untreated model mice.

DISCUSSION

Much evidence showed that an abnormal immune state, especially with activated T lymphocytes and overproduction of suppressive hematopoietic cytokines played an important role in the generation of AA[1,2]. IFN γ and TNF α, mainly produced by lymphocytes, are two cytokines involved in the development of AA. Most of AA patients have high expression of IFN γ, TNF α, and macrophage-inflammatory-protein in their peripheral lymphocytes[6]. *In vitro* IFN γ and TNF α could inhibit the hematopoietic stem/progenitor cells which were responsible for the balance of blood cells, even inducing them to apoptosis[7]. So, immunity suppressive drugs such as anti-lymphocyte-IgG, anti-thymocyte-IgG, and cyclosporin A have been used for treating AA with good effects. Both of these agents have an inhibitory effect on $CD8^+$ T lymphocytes.

Shibata *et al* found that histamine type Ⅱ receptors (H_2R) existed on T lymphocytes, and H_2R antagonist Cim could decrease H_2R number and inhibit lymphocytes function[3]. The effect of Cim on the functioning of lymphocytes derived from AA is unknown. The immunity-derived AA mice model was used here to substitute for human AA patients. Our unpublished data and reports of some researchers have revealed that this AA model had similar symptoms and blood and bone marrow changes as in AA patients[5]. In the present study, the spleen lymphocytes from AA mice produced higher concentration of IFN γ than those from irradiated and control mice, correlating well with the results of others[8]. The dynamic pattern of TNF α was similar to IFN γ in our study. Yao and Li reported that the expression of TNF α mRNA in AA mice were lower than the irradiated and control group, although the plasma levels of TNF α was high[9]. The reason underlying the difference between the results remains to be elucidated. Previous evidences showed that the splenocytes from AA mice inhibited the normal hematopoiesis via overproduction of suppressive cytokines such as IFN γ and TNF α, which participated in the formation of AA. Our results revealed for the first time that $H_2$2R antagonist Cim could not only reduce the production of IFN γ by AA splenocytes, but also that of TNF α. These findings are suggestive for such a treatment of AA. Recently, Wang *et al* found that Cim 10 μmol/L could partially block the inhibitory effects toward normal hematopoiesis by $CD8^+$ T lymphocytes from AA patients[4,11]. In this study, it has been identified for the first time that H_2R antagonist Cim could decrease the production of IFN γ and TNF α in AA mice. This suggests that Cim may be potentially effective in the treatment of AA in human.

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西咪替丁抑制再生障碍性贫血小鼠脾细胞产生干扰素γ和肿瘤坏死因子α

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关键词 西咪替丁; 再生障碍性贫血; 肿瘤坏死因子; 干扰素 Ⅱ型; 酶联免疫吸附测定

目的: 研究西咪替丁对免疫介导再生障碍性贫血小鼠淋巴细胞产生 IFN γ 和 TNF α 的影响. **方法**: 亚致死量照射后输注异种淋巴细胞构建再障小鼠, 用 LPS 或 PHA-P 刺激脾脏细胞产生 TNF α 或 IFN γ, 夹心 ELISA 法检测诱生的 IFN γ 浓度, 用 L929 细胞毒法测定 TNF α 水平. **结果**: (1) 再障鼠淋巴细胞诱生的 IFN γ 和 TNF α 浓度分别为(137±36) ng/L and (6±3) μg/L, 均高于单纯放射组及对照组; (2) 用西咪替丁处理淋巴细胞后, 再障小鼠淋巴细胞产生的 IFN γ 和 TNF α 水平下降, 分别为(14±8) ng/L 和 (2.7±0.6) μg/L. **结论**: 西咪替丁能有效减少再障小鼠脾淋巴细胞 IFN γ 和 TNF α 的产生.

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