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# The Effect of Vitamin A on Secretion of IFN-γ and IL-4 in A549 Cells Induced by *Mycoplasma Pneumoniae*

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**Summary:** In order to investigate the effect of vitamin A (VA) on the secretion of IFN-γ and IL-4 in *Mycoplasma Pneumoniae* (MP)-induced A549 cells, A549 cells were co-cultured with MP for different time lengths and then the levels of IFN-γ and IL-4 in the cell culture supernatants were detected before and after treatment with different concentrations of VA by using the enzyme-linked immunosorbent assay ( ELISA). The results showed that the level of IFN-γ and IL-4 in the supernatants of MP-induced A549 cells was much higher than that in non-induced cells ($P$<0.01). After application of VA, IL-4 level was not increased until the concentration of VA was up to $0.5\times10^{-5}$ mol/L ($P$<0.01). However, with concentration of VA increased up to $1\times10^{-4}$ mol/L, IL-4 was significantly suppressed ($P$<0.01). It was concluded that MP could induce the secretion of IFN-γ and IL-4 in A549 cells. VA could inhibit the secretion of IFN-γ and increase the IL-4 level in MP-induced A549 cells. However, high concentration of VA had an inhibitory effect on the secretion of IL-4 as well as on the IFN-γ. These data provided a theoretical basis for the application of VA in MP pneumonia in the clinical practice.
**Key words:** *Mycoplasma pneumoniae*; vitamin A; IFN-γ; IL-4

*Mycoplasma pneumoniae* (MP) causes infection of not only respiratory system but also other important organs. It was found to contribute to the development of asthma in children[1]. The mechanism of MP infection is not clear so far. Some studies showed that low Th1-cell-mediated immunity and relatively high Th2-cell-mediated immunity were associated with MP pneumonia[2-4]. IFN-γ is a representative cytokine secreted by Th1 cells, whereas IL-4 is a main cytokine from Th2 cells. In clinical practice, the levels of IFN-γ and IL-4 are believed to indirectly reflect the change of Th subsets. It is well documented that vitamin A (VA) can maintain the integrity of mucous membranes, enhance resistance to diseases[5], regulate the polarization mode of Th1/Th2 cytokines[6], and has anti-infection effect. Although VA has been extensively used in clinical practice, it was reported that pneumonia might be aggravated by administration of VA. In this study, the effect of VA on secretion of IFN-γ and IL-4 in MP-induced A549 cells was investigated. The mechanism of VA working against MP infection has been discussed.

## 1 MATERIALS AND METHODS

### 1.1 Materials

MP (ATCC15531 strain) was from the Department of Microbiology, Wuhan University, Wuhan, China. A549 cells were kindly provided by the Institute of Virology, Wuhan University, China. VA was purchased from Sigma Co. Ltd., USA. PPLO medium was bought from Difico Co., USA. Horse serum and RPMI1640 culture medium were procured from Hyclone Co., USA. Fetal calf serum was from Gibco Co., USA. Yeast extracts were from Oxoid Co., USA. IFN-γ and IL-4 ELISA kit were products of Jingmei Biotech Co., China. Other reagents were of analytical grade.

### 1.2 The Culture of MP

Frozen MP was seeded into PPLO liquid medium and incubated at 37 °C in an atmosphere of 5% $CO_2$ and saturation of nitrogen. When the color of the medium changed from red to yellow, passage was performed. MP at exponential phase of growth was harvested and quantified by colony-formine unit (CFU). The collected bacterial fluid was centrifuged for 20 min at 4°C and then the precipitate was washed three times with phosphate-buffered saline (PBS, pH 7.2). After the precipitation, appropriate RPMI1640 was added to obtain bacterial suspension of $1\times10^9$ CFU MP/mL for later use.

### 1.3 The Culture of A549 Cells

A549 cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum at 37°C in the atmosphere of 5% $CO_2$ and saturated humidity. The cells were collected at the exponential phase and the cell concentration was adjusted to $1\times10^6$ cells/mL with RPMI1640 containing 5% fetal calf serum. A549 cells were seeded into 24-well plates, with each well having 0.5 mL medium, and then cultured at 37°C in 5% $CO_2$.

### 1.4 Grouping

On the basis of the results of preliminary experi-

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ment, A549 cells were divided into the following five groups: control group, in which no stimulant was added into the medium; MP group, in which cells were co-cultured with $1\times10^7$ CFU/mL of MP [multiplicity of infection (MOI )1:20, diluted by using RPMI1640 with 2% fetal calf serum]; groups A, B and C, in which different concentrations of VA were added into the cell medium ($1\times10^{-6}$ mol/L VA in group A, $0.5\times10^{-5}$ mol/L VA in group B, and $1\times10^{-4}$ mol/L VA in group C). MOI in these three groups was 1:20. The five groups each had 4 wells with each containing 1 mL medium. Cells in the groups were cultured for 2, 4, 8, 16 and 24 h respectively at 37°C in the atmosphere of 5% $CO_2$ and saturated humidity. All examinations were performed four times at each time point.

### 1.5 The Detection of IFN-γ and IL-4

The cell culture supernatant was harvested for each group. The levels of IFN-γ and IL-4 were detected according to the instruction of ELISA kit.

### 1.6 Statistical Analysis

Experimental data were expressed as $\bar{x}\pm s$. One-factor analysis of variance, SNK and DunnettT3 were performed by using SPSS12.0 software package. A $P<0.05$ was considered to be statistically significant.

## 2 RESULTS

### 2.1 Effect of MP and VA on IFN-γ Level in A549 Cells

IFN-γ level in MP group as compared with control group started to increase at the 2nd h, was substantially elevated at the 8th h and reached a peak at the 24th h. Upon addition of different concentrations of VA, IFN-γ level was lowered to various degrees (table 1). Significant differences were noted in IFN-γ level among the 5 groups at the 16th h ($F$=1785.91, $P<0.01$ for all). The results suggested that MP could markedly stimulate the secretion of IFN-γ in A549 cells and with the increase of the concentration of VA, IFN-γ level was decreased gradually.

**Table 1 Effect of MP and VA on IFN-γ level in A549 cells ($\bar{x}\pm s$, pg/mL, MP 1∶20)**

| Groups | 2 h | 4 h | 8 h | 16 h | 24 h |
|---|---|---|---|---|---|
| Control | 9.533±1.569 | 12.340±1.021 | 13.903±1.197 | 16.390±1.187 | 22.318±2.037 |
| MP | 11.091±1.019 | 23.568±1.017 | 99.660±2.032* | 116.791±2.133* | 119.000±3.314* |
| A | 11.404±1.195 | 26.065±1.028 | 70.968±2.278 | 87.182±2.036# | 82.510±1.568 |
| B | 7.974±1.248 | 29.493±1.192 | 74.714±2.277 | 79.078±2.162# | 81.888±0.625 |
| C | 11.404±1.195 | 17.640±1.568 | 47.893±1.245 | 51.325±1.196# | 57.248±1.017 |

*$P<0.01$ as compared with the control group; #$P<0.01$ as compared with MP group

### 2.2 Effect of MP and VA on IL-4 Level in A549 Cells

IL-4 levels in MP group at different time points showed changes similar to those of IFN-γ, starting to increase at the 4th h and further increasing with time and reaching a peak at the 24th h. When $0.5\times10^{-5}$ mol/L of VA was added, IL-4 level in A549 cells in the group B was increased as compared with that in control group and MP group. IL-4 level in group C was much lower than that in groups A, B and MP (table 2). There were significant differences in IL-4 level among the 5 groups at 16-h time point ($F$=452.97, $P<0.01$). No significant difference was found in IL-4 level between MP group and group A as revealed by multiple-group comparison ($P>0.05$). IL-4 level was further increased after treatment with VA of $0.5\times10^{-5}$ mol/L.

**Table 2 Effect of MP and VA on IL-4 level in A549 cells ($\bar{x}\pm s$, pg/mL, MP 1∶20)**

| Groups | 2 h | 4 h | 8 h | 16 h | 24 h |
|---|---|---|---|---|---|
| control | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 |
| MP | 0.000±0.000 | 0.142±0.284 | 21.320±0.996* | 24.978±1.988* | 29.553±1.540* |
| A | 0.000±0.000 | 0.284±0.328 | 24.693±2.015 | 24.060±2.305 | 31.078±2.992 |
| B | 0.142±0.284 | 15.210±0.996 | 25.590±3.070 | 34.885±1.763 | 33.515±5.808 |
| C | 0.000±0.000 | 1.789±0.997 | 4.840±2.114 | 15.515±2.705 | 15.517±3.647 |

*$P<0.01$ as compared with the control group

### 2.3 Effect of VA and MP on the Ratio of IFN-γ and IL-4

The ratio of IFN-γ and IL-4 was compared among the 5 groups at the 16th h, with the differences being statistically significant ($F$=38.78, $P<0.01$). There was no significant difference in the ratio of IFN-γ and IL-4 between group A and group C as shown by multi-group comparison ($P>0.05$). IL-4 was lower in groups A and B than in MP group and when high-dose of VA was added, the ratio was increased and the value was similar to that in group A (table 3).

**Table 3 Effect of VA and MP on ratio of IFN-γ and IL-4 ($\bar{x}\pm s$, MOI 1∶20)**

| Groups | VA (mol/L) | Time（h） | IFN-γ/IL-4 | $F$ | P |
|---|---|---|---|---|---|
| MP | | 16 | 4.693±0.297 | | |
| A | $1\times10^{-6}$ | 16 | 3.642±0.260 | 38.78 | <0.01 |
| B | $0.5\times10^{-5}$ | 16 | 2.269±0.058 | | |
| C | $1\times10^{-4}$ | 16 | 3.374±0.936 | | |

## 3 DISCUSSION

VA is a fat-soluble vitamin and an essential nutrient for the formation of the bone, the growth and development of the human body, the maintenance of normal vision and immunity. As a common nutritional supplement, VA is widely used in food and clinical practice, especial in the treatment of respiratory diseases. Meta-analysis showed that VA reduced the mortality of measles to 64% and mortality of pneumonia to 67%[7]. In recent years, trials of high-dose VA for the treatment of pneumonia and lower respiratory tract infection conducted in community hospitals yielded controversial results concerning the role of VA. Some studies showed that high-dose VA may hamper the rehabilitation from pneumonia[8-10], while others suggested that VA could enhance children's immune function, improve the anti-infection capacity of the digestive and respiratory system[5]. Because some of these studies were carried out in animals, or in pneumonia subjects in which pathogens were not definitely identified, further investigation was needed to clarify the role of VA under different conditions. In this study, the effects of VA on MP pneumonia were investigated by detecting the cytokines in MP-induced lung epithelial A549 cells to provide experimental basis for the proper use of VA in the treatment of MP pneumonia.

MP pneumonia was associated with the cellular immune dysfunction. Pro-inflammatory mediators, such as IFN-γ, IL-2, IL-12 and TNF-α were secreted by Th1 cells. These cytokines could enhance the cytotoxicity of killer cells and cell-mediated immune response. Th2 cells secrete IL-4, IL-5, IL-6, IL-10, which promote the formation of antibodies, and mediate humoral immune responses. Under normal circumstances, Thl/Th2 could regulate each other and thereby maintain normal immunity. It has been reported that MP pneumonia was attributable to Thl/Th2 imbalances, which was manifested by significantly high levels of IL-4 and IL-4/IFN-γ and dominance of Th2 cells in immune response[2-4]. Th0 can be polarized and transformed to Th2 cells in MP pneumonia, thereby alleviating the MP-induced inflammatory reaction, enhancing resistance and removing the pathogen.

VA can regulate the polarization mode of Th1/Th2 cytokines and increase the amount of the Th2 cytokines such as IL-4[6], which can provide a further anti-infective effect. On basis of *in vitro* cell experiments, Stephensen *et al*[11] indicated that VA influenced Th0 cells via the RXR (retinol-X receptor) pathway, inhibiting the Th1 gene expression and promoting Th2 gene expression. Iwata *et al*[12] also suggested that VA promoted the production of Th2 cells by directly inhibiting Th1 cells via the RAR (retinoic acid receptor). In this study, we found that the levels of IFN-γ and IL-4 secreted by MP-induced A549 cells changed with administration of VA. VA lower than $0.5\times10^{-5}$ mol/L could inhibit the secretion of IFN-γ, increase the secretion of IL-4 and thereby reduce the ratio of IFN-γ and IL-4. This finding was consistent with previous study, which indicated that VA could enhance the secretion of Th2 cytokines[6]. However, it was found that VA of $1\times10^{-4}$ mol/L could inhibit the secretion of both IL-4 and IFN-γ and increase the ratio of IFN-γ and IL-4. The results suggested that it was crucial to control the dose of VA in the treatment of pneumonia. Optimal concentration of VA could enhance body resistance to inflammation, speed up the removal of pathogens, and promote rehabilitation. High dose of VA had harmful effects on rehabilitation of a series of respiratory diseases rather than enhance the protective effects of Th2 cytokines[13]. Furthermore, a high-dosage of VA could inhibit the secretion of IFN-γ and result in further reduction of Th1-cellular immune function. On the other hand, because Th1-mediated response is caused by resistance of the host cell to infection, the reduced Th1-cellular immune function hinders the recovery of host from viral infection[13]. Previous research also showed that VA aggravated respiratory syncytial virus pneumonia or community acquired pneumonia[14]. In addition, because IL-4 can induce the antibody conversion in B cells and produce IgE, strong response in the airway may result.

Hoek *et al*[15] discovered that 2 h after MP infection, the expression of IL-4 in murine mast cells were increased, with IL-4 level reaching a peak at the 4th h and dropping to baseline level after 24 h. More recently, Hoek *et al*[16] found that adherent MP could induce degranulation of mouse mast cells, promote the synthesis of IL-4 mRNA and produce IL-4. Ten Hacken *et al*[17] also confirmed the presence of high level IL-4 in serum in the patients with MP pneumonia. Other researchers found that, in asthma children with MP pneumonia, supplement of VA could enhance the levels of Th2-type cytokines and increase the secretion of IL-4, indicating that supplement of VA might well increase the airway hyper-responsiveness and worsen inflammation in children with MP-induced asthma. In the past, it was generally accepted that supplement of VA could improve immunity. However, this notion is not necessarily true as it fails to consider the combined effects of a number of diseases under different conditions. Therefore, for juvenile MP pneumonia complicated with asthma or for pneumonia with unidentified pathogens, VA should not be given until the cause of the disease is fully understood.

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