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Use of flow cytometry to investigate the cytokine response pattern in infants with respiratory syncytial virus infection and bronchiolitis

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Abstract： Objective：To investigate the cytokine response pattern (IL-4/IFN-γ) in infants with RSV infections and bronchiolitis during the acute phase. Methods：Four-color flow cytometry was used to measure intracellular IL-4 and IFN-γ expressions in peripheral blood CD3+ and CD8+ lymphocytes from RSV-infected and bronchiolitis infants. Serum IL-4 and IFN-γ levels were also determined. Results：RSV-infected and bronchiolitis infants showed no statistical differences from not-RSV-infected or pneumonia infants and control in the frequency of IL-4 and IFN-γ expressions in CD3+CD8- lymphocytes，showed no obvious Th1/Th2 imbalance，while IFN-γ was expressed much more frequently in CD3+CD8+ lymphocytes. Systematically，RSV-infected and bronchiolitis infants showed much lower levels of serum IL-4 and IL-4/IFN-γ ratios and much higher serum IFN-γ levels than control. However，there were no statistical differences in the above three indices between RSV-infected and not-RSV infected infants or between bronchiolitis and pneumonia infants，except that bronchiolitis infants had a higher level of serum IFN-γ than pneumonia infants statistically. Conclusions：There is no type-2 cytokine response predominance in the acute phase of RSV infection and bronchiolitis. IL-4 production is suppressed and IFN-γ production upregulated，the latter being most prominent in bronchiolitis infants.

Key words： Respiratory syncytial virus(RSV)，Bronchiolitis，IL-4/ IFN-γ，Flow cytometry
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INTRODUCTION

Respiratory syncytial virus (RSV) is the most important pathogen of lower respiratory infection in infants and children. Primary infection in young infants usually manifests as bronchiolitis or pneumonia. Many studies have shown that RSV bronchiolitis is associated with the development of recurrent wheezing and childhood asthma，in which an imbalance of Th function，favoring Th2 predominant response，is believed to be very important (Sigurs et al.，2000；Kneyber et al.，2000). This phenomenon leads to the hypothesis that RSV bronchiolitis is the result of predominant type 2 cytokine response. Animal studies have revealed that RSV can stimulate a Th2 lymphocyte response. The strongest evidence came from the animal model of formalin-inactivated virus (FI-RSV)-enhanced disease，in which the selective activation of Th2 cells and predominant type 2 cytokines appear to play an important role in determining the disease outcome of RSV infection (Waris et al.，1996).

However，the selective differentiation of Th2 cells can be influenced by a variety of factors，including genetic background (Hussell et al.，1998). The conclusion by extrapolating from the murine model of FI-RSV enhanced disease to human RSV infection may be incorrect. Therefore，whether a similar immunopathological mechanism plays a role in naturally occurring human RSV bronchiolitis remains uncertain.

This study aimed to determine the cytokine response pattern in infants with RSV infection and bronchiolitis based on cytokine profiles of CD4+ and CD8+ lymphocytes by intracellular cytokine staining technique and flow cytometry combined with serum IL-4 and IFN-γ determination.

SUBJECTS AND METHODS

Patients and samples

Fifty infants aged 2 months to 2 years，ad-

mitted to the Children's Hospital, College of Medicine Zhejiang University from Oct. 2000 to Apr. 2001, were included in the study. They were diagnosed as bronchiolitis (defined as diffuse wheezing and hyperinflation but infiltrate-free chest radiograph, $n=28$ with 23 boys and 5 girls) or pneumonia ($n=22$ with 17 boys and 5 girls). This study was approved by the Ethics Committee of the hospital. All the patients had been having the problem for less than 5 days, and had no history of administration of glucocorticoids in the past two weeks. Upon admission, samples of sera for determination of IL-4 and IFN-γ, heparinized blood for intracellular cytokine profile study and nasopharyngeal aspiration for RSV antigen detection were simultaneously collected. A control group consisted of 30 infants with 23 boys and 7 girls, aging from 4 months to 24 months. They were hospitalized for minor surgery without allergic and immunological diseases and had no history of respiratory infections and medication in the past two weeks.

RSV diagnosis

Nasopharyngeal aspirates were collected using an 8-French soft catheter attached to a valve-operated trap connected to an electric suction device, and transported to laboratory as soon as possible. After centrifugation, the cell pellet was resuspended and one drop of cell suspension placed onto precleaned slides; allowed slide to air dry and fixed it in chilled acetone. Direct immunofluorescence (light diagnostics respiratory panel I DFA, Chemicon International, CA) was utilized to identify the RSV antigen in these specimens. The fluorescein-labeled monoclonal antibody will specifically bind to the RSV antigen in the cell, showed apple-green fluorescence by fluorescence microscopy. Thirty-one of above patients were diagnosed as RSV positive, the other 19 patients as RSV negative.

Cytokine assays

IL-4 and IFN-γ levels in sera from 27 bronchiolitis and 28 RSV-infected infants were measured using commercially available ELISA kits (DIACLONE, France). The detailed protocols were in accordance with the manufacture's instructions. Reproducibility of the ELISAs on repeated assays was within 10%, and every measurement was done in duplicate.

Intracellular cytokine staining and detection

1. in vitro lymphocyte culture and cytokine induction: Peripheral blood mononuclear cells (PBMCs) were separated from fresh heparinized blood via Ficoll-Paque density-gradient centrifugation. Cells were stimulated in a 24-well plate for 6 hours at 37℃ with 7% CO_2 by 10 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma) and 1 μg/ml of ionomycin (Sigma). Ten μg/ml of Brefeldin A (BFA, Sigma) was also added to disrupt Golgi function and allow cytokines to accumulate within the cytoplasm.

2. Cell surface marker and intracellular cytokine staining: The PMA/ionomycin-activated PBMCs were first labeled with APC-conjugated anti-CD3 and PerCP-conjugated anti-CD8 monoclonal antibodies. After permeabilization, the accumulated intracellular IL-4 and IFN-γwere stained by PE-conjugated anti-IL-4 and FITC-conjugated anti-IFN-γmonoclonal antibodies (Becton Dickinson, CA).

3. Flow cytometry: Surface phenotype and intracellular cytokine analysis were performed on a flow cytometer (FACSCalibur, Becton Dickinson, CA). A correlated analysis of the forward and right-angle scatters was used to establish the lymphocyte gate. The four-color analysis followed on obtaining 10 000 cells in lymphocytes per sample and CellQuest Software was used to analyze the data.

Statistical analysis

All the data in the study were expressed as means ± standard deviation ($\bar{x} \pm SD$). The non-parametric Kruskal-Wallis H test was first used for the comparison of the difference across the groups. If significant differences were identified, individual groups were compared by using the Mann-Whitney U-test. $P<0.05$ was considered to be significant.

RESULTS

IL-4 and IFN-γ expressions in CD3+CD8- and CD3+CD8+ lymphocytes from RSV-infected infants

Table 1 shows no statistical differences among RSV-infected, not-RSV-infected and control in the frequency of IL-4 and IFN-γ expressions in both CD3+CD8- and CD3+CD8+ lymphocytes, except that RSV-infected, as well

as not-RSV-infected infants, had a much higher frequency of IFN-γ expression in CD3+CD8+ lymphocytes than control.

Table 1 Effect of RSV infection on IL-4 and IFN-γ expressions in CD3+CD8- and CD3+CD8+ lymphocytes

Groups†		CD3+CD8−		CD3+CD8+	
		IL-4(%)	IFN-γ(%)	IL-4(%)	IFN-γ(%)
RSV+	($n=25$)	0.216±0.294	1.270±1.836	0.798±1.008	3.633±3.823*
RSV−	($n=18$)	0.262±0.239	1.711±1.901	1.263±1.319	4.892±4.283*
Control	($n=14$)	0.251±0.211	0.843±0.953	0.831±0.435	1.628±2.115
P		0.441	0.145	0.108	0.010

† RSV+: RSV-infected infants; RSV−: not-RSV-infected infants; * RSV+ or RSV− vs control: $P<0.05$; RSV+ vs RSV−: $P>0.05$.

IL-4 and IFN-γ expressions in CD3+CD8- and CD3+CD8+ lymphocytes from bronchiolitis infants

Table 2 shows no statistical differences among the bronchiolitis, pneumonia infants and control in the frequency of IL-4 expressions in both CD3+CD8- and CD3+CD8+ lymphocytes. CD3+CD8+ lymphocytes from bronchiolitis infants expressed IFN-γ much more frequently than control, but no difference existed between bronchiolitis and pneumonia infants.

Table 2 IL-4 and IFN-γ expressions in CD3+CD8- and CD3+CD8+ lymphocytes from bronchiolitis infants

Groups		CD3+CD8−		CD3+CD8+	
		IL-4(%)	IFN-γ(%)	IL-4(%)	IFN-γ(%)
Bronchiolitis	($n=22$)	0.179±0.225	1.925±2.372	0.769±0.760	5.270±4.992*
Pneumonia	($n=21$)	0.293±0.306	0.963±0.902	1.227±1.447	2.997±2.241
Control	($n=14$)	0.251±0.211	0.843±0.953	0.831±0.435	1.628±2.115
P		0.264	0.362	0.506	0.018

* bronchiolitis vs. control: $P<0.05$; bronchiolitis vs. pneumonia: $P>0.05$.

Serum IL-4 and IFN-γ levels in RSV-infected infants

Table 3 shows that RSV-infected infants had much lower levels of serum IL-4 and IL-4/IFN-γ ratios and much higher levels of serum IFN-γ than control. However, there were no statistical differences in above three indices between RSV-infected and not-RSV infected infants.

Table 3 Effect of RSV infection on serum IL-4, IFN-γ levels(pg/ml) and IL-4/IFN-γ ratios

Groups†	n	IL-4	IFN-γ	IL-4/IFN-γ ratio
RSV+	28	1.195±0.549	49.805±41.025	0.087±0.144
RSV−	19	1.048±0.514	44.857±55.794	0.112±0.149
Control	30	1.451±0.602	21.487±14.094	0.102±0.085
P		0.007*	0.024*	0.008*

† RSV+: RSV-infected infants; RSV−: not-RSV-infected infants; * RSV+ vs. control: $P<0.05$; RSV+ vs. RSV−: $P>0.05$.

Serum IL-4 and IFN-γ levels in bronchiolitis infants

Table 4 shows that bronchiolitis infants had much lower levels of serum IL-4 and IL-4/IFN-γ ratios and much higher levels of serum IFN-γ than the control. They also showed a higher level of serum IFN-γ than pneumonia infants statistically. But serum IL-4 level and IL-4/IFN-γ ratio in bronchiolitis infants were not statistically different from that in pneumonia infants.

Table 4 Serum IL-4 and IFN-γ levels (pg/ml) and IL-4/IFN-γ ratio in bronchiolitis infants

Groups	*n*	IL-4	IFN-γ	IL-4/IFN-γ ratio
Bronchiolitis	27	1.254±0.608	57.900±50.453	0.076±0.136
Pneumonia	20	1.085±0.564	34.177±39.138	0.112±0.149
Control	30	1.451±0.602	21.487±14.094	0.102±0.085
P		0.015*	0.004**	0.002*

* bronchiolitis vs control: $P<0.05$, bronchiolitis vs pneumonia: $p>0.05$; ** bronchiolitis vs control or pneumonia: $P<0.05$.

DISCUSSION

Previous studies on the relationship between RSV infection and cytokine response were usually based on the determination of cytokine proteins or their mRNA expression levels in cell culture supernant or body fluid such as blood. They had great diversity in methods adopted and also produced conflicting results (Bont et al., 2000; Roman et al., 1997; van Schaik et al., 1999). Furthermore, the measurement of cytokines in these specimens reflects the contribution of many types of cells and/or a physiological macro environment. Because different types of cells may produce the same cytokine, such methods may be inappropriate in evaluating Th1/Th2 balance.

The development of intracellular flow cytometric analysis makes it possible to evaluate single-cell cytokine profile, especially Th cell function. To our knowledge, there are to date only two studies involving the cytokine response pattern in human RSV infection defined by flow cytometry, with conflicting results (Bendelja et al., 2000; Brandenburg et al., 2000). Bendelja (Bendelja et al., 2000) reported a predominant type-2 response in infants with RSV infections, while another study conducted by Brandenburg (Brandenburg et al., 2000) showed that the responses were dominated by the production of IFN-γ and that only low levels of IL-4 were detectable.

Using four-color flow cytometry technique, we detected intracellular IL-4 and IFN-γ expressions of peripheral blood CD3+ and CD8+ lymphocytes. Because of the lack of constitutive expression of cytokines in resting lymphocytes in vitro, the intracellular cytokine flow cytometric analysis always need pre-stimulation with mitogens or specific antigens. PMA is a good candidate beause of its effectivity in stimulating the differentiation and proliferation of T lymphocytes and secretion of cytokines. However, our previous study (data unpublished) and that of some others (Sewell et al., 1997) revealed that CD4+ antigen may be downregulated to varying degree by PMA activation, and thus lead to an incorrect result of CD4+ phenotypic subsetting. The effect of PMA on CD8+ and CD3+ antigen expressions was much less (our previous study data unpublished). Therefore, we did cell surface marker analysis with both CD3+ and CD8+ monoclonal antibodies, and like Rudwaleit defined CD4+ lymphocyte indirectly by CD3+CD8- cells (Rudwaleit et al., 2000).

Our study showed that although both RSV-infected and bronchiolitis infants had only a slightly lower frequency of IL-4 expressions in CD3+CD8- and CD3+CD8+ lymphocytes with no statistical differences from control, they did show much lower serum IL-4 levels. However, no differences in serum IL-4 levels were found between RSV-infected and not-RSV infected infants or between bronchiolitis and pneumonia infants, indicating a general suppression of IL-4 production in lower respiratory infections.

In contrast to IL-4, the production of IFN-γ was upregulated. RSV-infected and bronchiolitis infants showed a much higher frequency of IFN-γ expression in CD3+CD8+ lymphocytes, and they also had much higher serum IFN-γ levels, especially bronchiolitis infants. The antiviral activity of IFN-γ had been well established. On the other hand, IFN-γ might also play a role in causing obstructed airway disease. Both protective and pathogenic roles were confirmed by an animal study wherein mice lacking IFN-γ developed more extensive inflammation of the airways than control, but exhibited less severe signs of airway obstruction (van Schaik et al., 2000).

Because of the decreased level of serum IL-4 and increased level of serum IFN-γ, the IL-4/IFN-γ ratio in RSV-infected and bronchiolitis

infants were much lower than the control, indicating type 1 cytokine response predominance systematically. Furthermore, no differences in IL-4 and IFN-γ expressions in CD3 + CD8- lymphocytes were found between the RSV-infected and not-RSV-infected infants or between bronchiolitis and pneumonia infants, showing no obvious Th1/Th2 imbalance in RSV infection and bronchiolitis. Additionally, CD3 + CD8 + lymphocytes from RSV-infected infants and bronchiolitis infants showed much more frequent IFN-γ expressions, with a type 1 cytokine response predominance again on single T-cell basis.

In summary, no type 2 cytokine response predominance was found during the acute phase of RSV infection and bronchiolitis, based on system level and intracellular cytokine analysis of single lymphocyte. IL-4 production was suppressed and IFN-γ production upregulated, the latter being most prominent in bronchiolitis, which might be associated with the development of bronchial obstruction in these infants.

References

Bendelja, K., Gagro, A., Bace, A., Lokar-Kolbas, R., Krsulovic-Hresic, V., Drazenovic, V., Mlinaric-Galinovic, G., Rabatic, S., 2000. Predominant type-2 response in infants with respiratory syncytial virus (RSV) infection demonstrated by cytokine flow cytometry. *Clin Exp Immunol.*, **121**(2): 332-338.

Bont, L., Heijnen, C. J., Kavelaars, A., van Aalderen, W. M., Brus, F., Draaisma, J. T., Geelen, S. M., Kimpen, J. L., 2000, Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one-year follow-up study. *Am J Respir Crit Care Med*, **161**(5): 1518 - 1523.

Brandenburg, A. H., Kleinjan, A., van HetLand, B., Moll, H. A., Timmerman, H. H., de-Swart, R. L., Neijens, H. J., Fokkens, W., Osterhaus, A. D., 2000, Type 1-like immune response is found in children with respiratory syncytial virus infection regardless of clinical severity. *J Med Virol*, **62**(2): 267 - 277.

Hussell, T., Georgiou, A., Sparer, T. E., Matthews, S., Pala, P., Openshaw, P. J., 1998. Host genetic determinants of vaccine-induced eosinophilia during respiratory syncytial virus infection. *J Immunol*, **161**(11): 6215 - 6222.

Kneyber, M. C. J., Steyerberg, E. W., de Groot, R., Moll, H. A., 2000. Long-term effects of respiratory syncytial virus (RSV) bronchiolitis in infants and young children: a quantitative review. *Acta Paediatr*, **89**(6): 654 - 660.

Roman, M., Calhoun, W. J., Hinton, K. L., Avendano, L. F., Simon, V., Escobar, A. M., Gaggero, A., Diaz, P. V., 1997. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. *Am J Respir Crit Care Med*. **156**(1): 190 - 195.

Rudwaleit, M., Yin, Z., Siegert, S., Grolms, M., Radbruch, A., Braun, J., Sieper, J., 2000. Response to methotrexate in early rheumatoid arthritis is associated with a decrease of T cell derived tumour necrosis factor α, increase of interleukin 10, and predicted by the initial concentration of interleukin 4. *Ann Rheum Dis*, **59**(4): 311 - 314.

Sewell, W. A., North, M. E., Webster, A. D. B., Farrant, J., 1997. Determination of intracellular cytokines by flow-cytometry following whole-blood culture. *J Immunol Methods*, **209**(1): 67 - 74.

Sigurs, N., Bjarnason, R., Sigurbergsson, F., Kjellman, B., 2000. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am J Respir Crit Care Med*, **161**(5): 1501-1507.

van Schaik, S. M., Tristram, D. A., Nagpal, I. S., Hintz, K. M., Welliver, R. C., Welliver, R. C. 1999. Increased production of IFN-γ and cysteinyl leukotrienes in virus-induced wheezing. *J Allergy Clin Immunol*, **103**(4): 630 - 636.

van Schaik, S. M., Obot, N., Enhorning, G., Hintz, K., Gross, K., Hancock, G. E., Stack, A. M., Welliver, R. C., 2000. Role of interferon gamma in the pathogenesis of primary respiratory syncytial virus infection in BALB/Cc mice. *J Med Virol*, **62**(2): 257 - 266.

Waris, M. E., Tsou, C., Erdman, D. D., Zaki, S. R., Anderson, L. J., 1996. Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. *J. Virol*, **70**(5): 2852 - 2860.