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Development of Interleukin 6 and Tumor Necrosis Factor Alpha Activity in Nasopharyngeal Secretions of Infants and Children during Infection with Respiratory Syncytial Virus

KAZUKO MATSUDA,[1] HIROYUKI TSUTSUMI,[1]* YOSHITAKA OKAMOTO,[2] AND CHUNZO CHIBA[1]

Department of Pediatrics, Sapporo Medical University School of Medicine, Sapporo,[1] and Department of Otolaryngology, Akita University School of Medicine, Akita,[2] Japan

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Cytokine (interleukin 6 [IL-6] and tumor necrosis factor alpha [TNF-α]) activity in nasopharyngeal secretions of 21 infants and children (19 days to 16 months old) infected with primary respiratory syncytial virus was determined by an enzyme-linked immunosorbent assay. IL-6 and TNF-α were detectable in 100% (21 of 21) and 67% (14 of 21) of cases during the course of infection, respectively. Generally, TNF-α activity was high in the acute phase and declined thereafter, sometimes to undetectable levels. IL-6 activity was also highest in the acute phase and declined thereafter in infants younger than 5 months, while in patients older than 5 months, it increased during the course of the disease to peak in the early convalescent phase. These observations suggest that inflammatory cytokines are produced in vivo in infants and children in response to primary respiratory syncytial virus infection and may be involved in disease pathogenesis. However, the mechanism of induction of cytokines may be different for infants and children in different age groups.

Respiratory syncytial virus (RSV) is the leading cause of severe lower respiratory tract disease in infants and young children (7, 18). The pathogenesis of RSV-induced bronchiolitis is poorly understood (6, 19). The mechanisms of injury of the human lung in RSV infection are thought to be secondary to viral replication in airway epithelial cells as well as to the local immune response (6, 7, 19). Alveolar macrophages are thought to be a possible target for RSV infection as well as for infection by other respiratory viruses (9, 20) and may be engaged as the first line of defense (3, 14, 15, 17), since they produce a wide range of immunological mediators such as tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), IL-6, and IL-8 in response to RSV infection (3, 16, 17). These studies were done in vitro with macrophages derived from either normal adults or adults with RSV infection. RSV replication and expression of IL-1 and TNF-α in alveolar macrophages were confirmed at the cellular level in a recent study of infants with RSV infection (8). The production of TNF-α and IL-6 in response to intranasal inoculation of high-dose RSV has been demonstrated in vivo in mice (4). However, cytokine activity in the site of infection in infants and children during primary RSV infection has not yet been quantitatively analyzed. The aim of the present study was to confirm the capacity of RSV infection in infants and children to induce TNF-α and IL-6 production in vivo and to clarify the kinetics of their development. In addition, we examined the relationship of cytokine production to the development of secretory immune response against RSV.

MATERIALS AND METHODS

Subjects. The study population consisted of 21 Japanese infants and children, 15 boys and 6 girls, ranging in age from 19 days to 16 months, with primary RSV infection. All subjects were inpatients of Sapporo Medical University Hospital between 1990 and 1993. Informed consent was obtained from each parent for the performance of the study. The nutritional and immunological status of each subject was considered normal. No premature babies were included. A detailed medical history was taken to exclude cases with possible recurrent RSV infection. Subjects over 6 months of age with high immunoglobulin G (IgG) antibody activity to RSV in acute-phase serum were also excluded from the study (data not shown). RSV infection was confirmed by virus isolation in tissue culture from nasopharyngeal secretion (NPS), and virus group determination of isolated viruses was carried out by enzyme-linked immunosorbent assay (ELISA) using several cross-reactive and subgroup-specific monoclonal antibodies, as described previously (12, 21). The clinical type of illness was determined by the definition of Mufson et al. (10).

Samples of NPS for initial diagnosis of RSV infection and for determination of cytokine and RSV-specific IgA antibody activity were obtained serially during the course of illness. NPS was soaked with 1 to 2 ml of minimal essential medium supplemented with 2% fetal calf serum. After centrifugation (1,500 × *g*, 10 min), the supernatant was frozen at −70°C until examination.

Total IgA concentrations of NPS were determined by ELISA with a standard IgA preparation, as described previously (23).

Cytokine assays. The determinations of IL-6 and TNF-α activity in NPS were carried out in duplicate with commercially available ELISA kits (Quantikine; R & D System, Inc.). Standard curves were prepared for all ELISAs. The detection limits of the tests were 10 and 50 pg/ml for IL-6 and TNF-α, respectively.

Cells and viruses. The RSV Long strain (prototype group A) and 58-17 field strain (group B), which was isolated in 1983 in Sapporo, Japan, were prepared and propagated in HEp-2 cell culture monolayers (12).

Assay for antibody to RSV. RSV-specific IgA antibody activity in NPS samples was determined by tissue culture ELISA as described previously (24). The RSV Long or 58-17 strain was used as the solid-phase antigen depending on the group (A or B) of RSV which was isolated from subjects. Acetone-fixed virus-infected or mock-infected HEp-2 cell plates were blocked with 1% bovine serum albumin (BSA)-phosphate-buffered saline (PBS) for 2 to 3 h. One-tenth-milliliter NPS samples which had been serially twofold diluted with 0.2% BSA-PBS were added in duplicate to the wells, and the wells were incubated for 1 h at 37°C. After washing the plates, peroxidase-labeled goat anti-human IgA (MBL, Nagoya, Japan) was added. After incubation for 1 h at 37°C, substrate was added to each well. Specific absorbance was calculated by subtracting the mean absorbance of two wells with uninfected cells from the mean absorbance of two wells with virus-infected cells. The ELISA titers in NPS samples were expressed as the highest dilution that gave a specific absorbance of more than 0.1.

The cytokine activity and IgA antibody ELISA titer in NPS samples were uniformly adjusted to a total IgA content of 0.1 mg/ml (11) because the thicknesses of the NPS samples obtained were different.

Geometric mean titers (GMT) for patients in different age groups were determined. For the purpose of calculating the GMT, specimens with cytokine levels under the limit of detection were given a value of one-half of the levels of the limit of detection. Statistical comparison between groups was done with the Wilcoxon test.

* Corresponding author. Mailing address: Department of Pediatrics, Sapporo Medical University School of Medicine, Chuoku S-1, W-16, Sapporo 060, Japan. Phone: 011 81 11 611 2111. Fax: 011 81 11 611 0352.

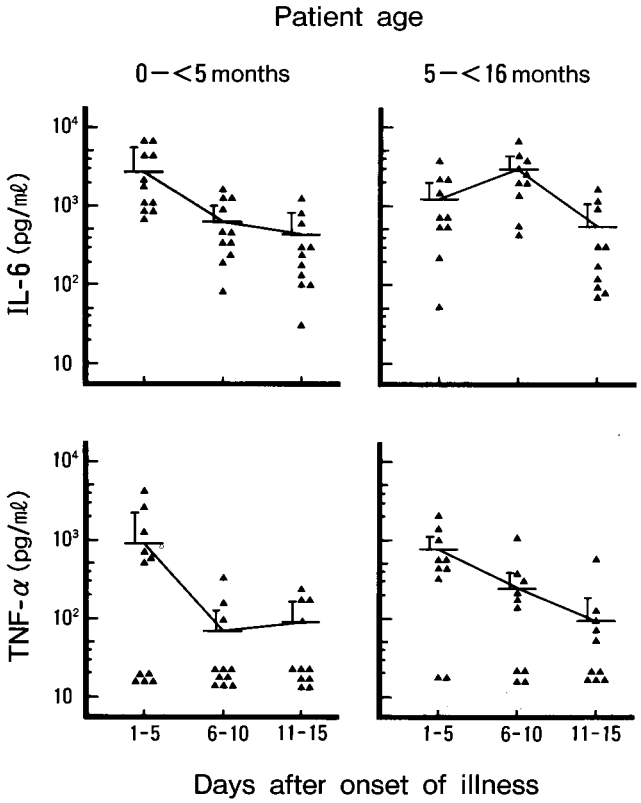

FIG. 1. Temporal pattern of cytokine (IL-6 and TNF-α) levels in NPS during primary infection with RSV. Patients were divided into two groups by age at infection, 0 to <5 months and 5 to 16 months. Cytokine levels were corrected to a total IgA content of 0.1 mg/ml. Bars indicate GMT + standard deviations.

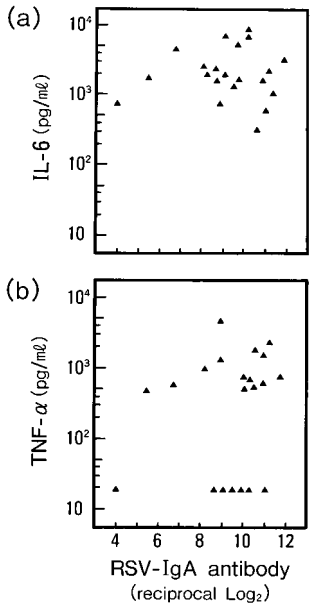

FIG. 2. Correlation between the activity of NPS ELISA anti-RSV IgA antibody in the middle convalescent phase and peak activities of NPS IL-6 (a) or TNF-α (b) during the course of primary infection with RSV. The NPS ELISA titers and cytokine levels were corrected to a total IgA content of 0.1 mg/ml.

RESULTS

Temporal kinetics of IL-6 and TNF-α activities. Patients were divided into two age groups, those younger and those older than 5 months of age. Of the 21 individuals, all were febrile, 5 had bronchitis, 14 had bronchiolitis, and 2 had pneumonia. In both age groups, bronchiolitis was the dominant type of illness. There were no significant differences in the proportions of the clinical types of illness between these two groups (data not shown). RSV infection in all patients was severe enough to require hospitalization. Of the 11 infants younger than 5 months, 9 and 2 were infected with group A and group B RSV, respectively. Of the 10 infants older than 5 months, 8 and 2 were infected with group A and group B RSV, respectively.

The temporal kinetics of the IL-6 and TNF-α activities observed in NPS during primary infection are shown in Fig. 1. In general, clinical symptoms of lower respiratory tract infection with RSV became worse until several days after the onset of illness, and in most instances, uneventful recovery occurred within 7 to 12 days (7). NPS samples collected for cytokine assay were divided into three groups on the basis of the interval between the onset of illness and sample collection: the acute (1 to 5 days), early convalescent (6 to 10 days), and middle convalescent (11 to 15 days) phases after the onset of infection.

IL-6 activity in patients younger than 5 months was high in the acute phase and declined thereafter. IL-6 activity in the acute phase (1 to 5 days; 2,740 ± 2,410 pg/ml) was significantly higher than that in the early (6 to 10 days; 640 ± 550 pg/ml) and middle (11 to 15 days; 480 ± 440 pg/ml) convalescent phases (all values are GMT ± standard deviation; $P < 0.01$ for both comparisons). On the other hand, in patients older than 5 months, the peak IL-6 activity in NPS was obtained during the early convalescent phase of infection. The activity in the early convalescent phase (6 to 10 days; 2,900 ± 2,030 pg/ml) was significantly higher than that in the acute phase (1 to 5 days; 1,280 ± 980 pg/ml) and that in the middle convalescent phase (11 to 15 days; 600 ± 630 pg/ml) (all values are GMT ± standard deviation; $P < 0.01$ for both comparisons).

There was no significant difference in the GMT of peak IL-6 activity between patients younger than 5 months and those older than 5 months ($P > 0.2$).

TNF-α activity was detected in 6 of 11 and 8 of 10 patients younger and older than 5 months, respectively. In almost all patients tested, TNF-α activity was high in the acute phase and declined thereafter. TNF-α activities of the acute phase (1 to 5 days) in the younger and older groups (870 ± 1,240 and 725 ± 610 pg/ml, respectively) were significantly higher than those observed in the early convalescent-phase samples (6 to 10 days; 69 ± 96 and 254 ± 300 pg/ml, respectively) (all values are GMT ± standard deviation; $P < 0.05$ for both comparisons).

Correlation of cytokine activity and RSV-specific IgA antibody activity. Peak RSV-specific IgA antibody titers in the samples collected during the middle convalescent phase (11 to 15 days) were adjusted to total IgA content and correlated with the peak IL-6 or TNF-α activity in each subject. No significant correlations were observed between the development of RSV-specific IgA antibody in the convalescent phase and the peak IL-6 activity during the course of infection ($r = 0.086$; $0.5 < P < 1$) (Fig. 2a). There was also no correlation between peak RSV-specific IgA antibody activity and peak TNF-α activity in NPS ($r = 0.350$; $0.2 < P < 0.4$) (Fig. 2b).

DISCUSSION

This is the first report of the quantitative analysis of development of IL-6 and TNF-α in infants and children with pri-

mary RSV infection. Significant IL-6 and TNF-α activity could be detected in the NPS of all and 67% of patients tested, respectively. The origin of these cytokines remains to be determined. They might be produced by alveolar macrophages (3, 8, 16, 17) and/or airway epithelial cells (3). TNF-α has been shown to induce the migration of neutrophils and mononuclear cells to the site of infection (13). TNF-α has been also shown to have antiviral activity (22). On the other hand, IL-6 has been shown to stimulate mucosal B lymphocytes (2). Thus, the increased production of these cytokines within several days postinfection suggests that they may be involved in disease pathogenesis early during infection as antiviral or immunoactive agents in the respiratory tract (3, 4, 17).

Of particular interest is the observation of differences in kinetics of development of IL-6 activity between infants younger than 5 months and that of older patients. IL-6 activity was high in the acute phase in the younger infants, while it peaked in the early convalescent phase in patients older than 5 months. No such tendency was seen in the kinetics of TNF-α activity.

Peak levels of both IL-6 and TNF-α activity in lung tissue from a mouse inoculated with high-dose RSV were detected at day 1 postinoculation (4). On the other hand, in lung tissue from an influenza virus-infected mouse, IL-6 activity continued to increase during the course of infection in spite of the decline of other cytokines such as TNF-α, IL-1, and gamma interferon (5). Our results with IL-6 in older infants seem to parallel those of the mouse model, although in this study, a bioassay, not the ELISA methods, was used for determination of cytokine activity.

IL-6 activity could be induced in older patients after the infection subsided, in response to another stimulus such as TNF-α, which is primarily induced by RSV infection. TNF has already been shown in vitro to be a potent inducer of IL-6 mRNA in bronchial epithelial cells as well as RSV infection (3).

Maternal factors may affect cytokine production in patients younger than 5 months. Preexisting (maternal) anti-RSV antibody may influence the IL-6 production in the acute phase of infection, possibly via the formation of immune complexes. In older patients, however, the enhancement of cytokine production with immune complexes might start only after indigenous RSV-specific antibody appears at the site of infection during the early convalescent phase. The effect of RSV-antibody complex on IL-8 release from granulocytes in vitro has been described recently (1).

It has been confirmed that IL-6 plays an important role in the terminal stages of mucosal immune responses, especially of the IgA antibody class, in the mouse model (2). However, in this study, no apparent positive correlation between peak IL-6 activity and the development of RSV-specific IgA antibody in respiratory tract was observed. The role of the development of cytokines in the respiratory tract on the outcome of RSV infection in infants and children remains to be elucidated.

REFERENCES

1. **Arnold, R., B. Werner, B. Humbert, H. Werchau, and W. Konig.** 1994. Effect of respiratory syncytial virus-antibody complexes on cytokine (IL-8, IL-6, TNF-α) release and respiratory burst in human granulocytes. Immunology **82:**184–191.
2. **Beagley, K. W., J. H. Eldridge, F. Lee, H. Kiyono, M. P. Everson, W. J. Koopman, T. Hirano, T. Kishimoto, and J. R. McGhee.** 1989. Interleukins and IgA synthesis: human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells. J. Exp. Med. **169:**2133–2148.
3. **Becker, S., J. Quay, and J. Soukup.** 1991. Cytokine (tumor necrosis factor, IL-6 and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. J. Immunol. **147:**4307–4312.
4. **Hayes, P. L., R. Scott, and J. Wheeler.** 1994. In vivo production of tumour necrosis factor-α and interleukin-6 in BALB/c mice inoculated intranasally with a high dose of respiratory syncytial virus. J. Med. Virol. **42:**323–329.
5. **Hennet, T., H. J. Ziltener, K. Frei, and E. Peterhans.** 1992. A kinetic study of immune mediators in the lungs of mice infected with influenza A virus. J. Immunol. **149:**932–939.
6. **Kimman, T. G., and F. Westenbrink.** 1990. Immunity to human and bovine respiratory syncytial virus. Arch. Virol. **112:**1–25.
7. **McIntosh, K., and R. M. Chanock.** 1990. Respiratory syncytial viruses, p. 1045–1072. *In* B. N. Fields and D. M. Knipe (ed.), Field's virology, 2nd ed. Raven Press, New York.
8. **Middula, F., A. Villani, J. R. Panuska, I. Dab, J. K. Kolls, R. Merolla, and R. Ronchetti.** 1993. Respiratory syncytial virus lung infection in infants: immunoregulatory role of infected alveolar macrophages. J. Infect. Dis. **168:**1515–1519.
9. **Morahan, P. S., J. R. Connor, and K. R. Leary.** 1985. Viruses and the versatile macrophages. Br. Med. Bull. **41:**15–21.
10. **Mufson, M. A., R. B. Belshe, C. Örvell, and E. Norrby.** 1988. Respiratory syncytial virus epidemics: variable dominance of subgroup A and B strains among children, 1981–1986. J. Infect. Dis. **157:**143–148.
11. **Murphy, B. R., B. S. Graham, G. A. Prince, E. E. Walsh, R. M. Chanock, D. T. Karzon, and P. F. Wright.** 1986. Serum and nasal-wash immunoglobulin G and A antibody response of infants and children to respiratory syncytial virus F and G glycoproteins following primary infection. J. Clin. Microbiol. **23:**1009–1014.
12. **Nagai, K., H. Tsutsumi, H. Yamazaki, S. Pattermadilok, and S. Chiba.** 1993. Three antigenic variant groups in human respiratory syncytial virus subgroup B isolated in Japan. Arch. Virol. **128:**55–63.
13. **Nathan, C., and M. Sporn.** 1991. Cytokines in context. J. Cell. Biol. **113:** 981–986.
14. **Neilson, K. A., and E. J. Yunis.** 1990. Demonstration of respiratory syncytial virus in an autopsy series. Pediatr. Pathol. **10:**491–502.
15. **Panuska, J. R., N. Cirino, F. Midulla, J. E. Despot, E. R. McFadden, and Y. T. Huang.** 1990. Productive infection of isolated human alveolar macrophages by respiratory syncytial virus. J. Clin. Invest. **86:**113–119.
16. **Panuska, J. R., M. I. Hertz, H. Taraf, A. Villani, and N. Cirino.** 1992. Respiratory syncytial virus infection of alveolar macrophage in adult transplant patients. Am. Rev. Respir. Dis. **145:**934–939.
17. **Panuska, J. R., F. Midulla, N. Cirino, A. Villani, I. A. Gilbert, E. R. McFadden, and Y. T. Huang.** 1990. Virus induced alteration in macrophage production of tumor necrosis factor and prostaglandin E_2. Am. J. Physiol. **259:**L396–L402.
18. **Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock.** 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. 2. Infection and disease with respect to age, immunological status, race and sex. Am. J. Epidemiol. **98:** 289–300.
19. **Pringle, C. R.** 1987. Paramyxoviruses and diseases, p. 51–90. *In* W. C. Russell and J. W. Almond (ed.), Molecular basis of virus disease. Cambridge University Press, Cambridge.
20. **Rogers, B. C., and C. A. Mims.** 1982. Influenza virus replication in human alveolar macrophages. J. Med. Virol. **9:**177–184.
21. **Tsutsumi, H., K. Nagai, K. Suga, Y. Chiba, S. Chiba, S. Tsugawa, and P. L. Ogra.** 1989. Antigenic variation of human RSV strains isolated in Japan. J. Med. Virol. **27:**124–130.
22. **Wong, G. H. W., and D. Goeddel.** 1986. Tumor necrosis factors α and β inhibit virus replication and synergize with interferons. Nature (London) **323:**819–820.
23. **Yamazaki, H., H. Tsutsumi, K. Matsuda, K. Nagai, P. L. Ogra, and S. Chiba.** 1994. Effect of maternal antibody on IgA antibody response in nasopharyngeal secretion in infants and children during primary respiratory syncytial virus infection. J. Gen. Virol. **75:**2115–2119.
24. **Yamazaki, H., H. Tsutsumi, K. Matsuda, K. Nagai, P. L. Ogra, and S. Chiba.** 1994. Respiratory syncytial virus group-specific antibody response in nasopharyngeal secretions from infants and children after primary infection. Clin. Diag. Lab. Immunol. **1:**469–472.