Natural Reinfection with Respiratory Syncytial Virus Does Not Boost Virus-Specific T-Cell Immunity

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ABSTRACT

To determine the role of respiratory syncytial virus (RSV)-specific cell-mediated immunity during natural reinfection, we investigated whether RSV-specific T-cell responses protect against reinfection and, subsequently, whether reinfection boosts virus-specific memory. In a cohort of 55 infants who were hospitalized for RSV bronchiolitis, RSV-specific lymphoproliferative responses in the peripheral blood were measured at three time-points: on admission, 4 wk after admission, and 1 y later, after the second winter season. Memory was defined as a stimulation index (SI) >2. During the second winter season, nasal secretions were collected in every case of a runny nose. Reinfection was diagnosed if immunofluorescence or PCR was positive for RSV. Virus-specific memory was found in one child on admission for primary RSV infection, whereas 4 wk later 44 infants (80%) had memory. Reinfection with RSV was found in 23 infants (43%) during the second winter season. After the second season, memory was found in 20 infants (38%). No differences in SI after the second winter season were found between

infants with and without reinfection (2.3 *versus* 2.1). However, a highly significant correlation was found between SI measured 4 wk after primary RSV infection and SI after the second winter season ($r = 0.40$, $p = 0.001$). In conclusion, RSV-specific T-cell responses did not provide protection against reinfection. Moreover, reinfection did not boost RSV-specific T-cell proliferation. To explain both findings, it is hypothesized that RSV-specific T cells fail to expand *in vivo* upon reinfection. (*Pediatr Res* 52: 363–367, 2002)

Abbreviations

RSV, respiratory syncytial virus
CMI, cell-mediated immunity
RT-PCR, reverse transcriptase PCR
TCID, tissue culture infection dose
LPR, lymphoproliferative response
IFN, interferon

RSV is one of the most important respiratory pathogens in infancy causing the majority of lower respiratory tract infections during the winter season. Hospitalization rates for RSV illness are 1–30 cases per 1000 infants <1 y of age (1–3). In hospitalized infants with RSV bronchiolitis, mechanical ventilation is required in 7–21% of cases (4–6). Mortality in RSV-infected infants with lower respiratory tract symptoms is <1% (7).

Reinfection with RSV occurs frequently and usually has a mild character with symptoms of uncomplicated upper respi-

ratory tract infection (8). Neutralizing antibodies induced by primary RSV infection appear to provide only partial protection for a limited period of time, which does probably not last until the subsequent RSV season (8, 9). Evidence that intact cell-mediated responses play a role in clearance of the virus and protection against reinfection was derived from animal studies (10). In humans, little information is available on the role of virus-specific CMI induced during primary infection in the protection against reinfection with RSV.

Currently, no vaccine for RSV is available. In the 1960s, a formalin-inactivated vaccine was used in infants (11). No protection against naturally acquired RSV was observed. In contrast, enhanced disease and increased mortality were observed during RSV infection after vaccination. CMI has been implicated in the pathogenesis of this phenomenon (12). Several strategies for safe

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and effective vaccination, including immunization with attenuated strains and subunit vaccines are under investigation at the moment (13–16). The immune response to an RSV vaccine should be protective, persistent, and not disease-enhancing upon subsequent contact with the virus. Similar to other vaccine strategies, such as measles and pertussis, it is conceivable that boosting is required to maintain virus-specific CMI. For this reason, it is important to study the characteristics of the memory response in relation to recurrent infection.

In this prospective follow-up study, we investigated the development over time of RSV-specific T-cell responses in a cohort of infants hospitalized for RSV bronchiolitis. The first aim of the study was to determine whether RSV-specific T-cell responses induced during primary RSV bronchiolitis protect against reinfection during the subsequent epidemic. The second aim of the study was to investigate whether virus-specific T-cell responses acquired during primary infection are boosted by natural reinfection with RSV.

METHODS

Study population. Infants were included during one winter epidemic in five hospitals in the Netherlands. Inclusion criteria were as follows: hospital admission, lower respiratory tract symptoms, age <13 mo, and positive immunofluorescence for RSV infection of epithelial cells in nasopharyngeal secretions. Lower respiratory tract symptoms were severe chest cough, wheezing, hoarseness, stridor, and shortness of breath (17), as well as cyanosis and apnea. Prematurely born infants with chronic lung disease and infants with wheezing illness before RSV bronchiolitis were not included. The study was approved by the medical ethical committees in all participating centers. Parents of subjects gave written informed consent.

Documentation of reinfection. During the second winter season, the occurrence of reinfection was studied. Parents received written and oral information during a home visit during the fall season before this part of this study. During the winter season, parents contacted the investigators (J.V.) in case of respiratory symptoms. To ascertain cooperation by parents, a telephone call was made every 3 wk. Within 48 h after onset of respiratory symptoms, home visits were made to perform nasal washes. No physical examination was performed.

RSV reinfection was confirmed by a commercially available direct immunofluorescence assay using FITC-labeled MAb (IMAGEN, DAKO, Glostrup, Denmark) and by RT-PCR. Reinfection was defined as a positive immunofluorescence or PCR on nasal washings.

RT-PCR. RNA extraction was performed according to the method described by Boom *et al.* (18). Briefly, 10–100 μ L respiratory specimen was mixed with 900 μ L lysis buffer and 50 μ L silica and incubated for 10 min at room temperature to bind the nucleic acid to the silica particles. Unbound material was then removed by several washing steps. The RNA was subsequently eluted in 100 μ L 40 ng/ μ L polyA RNA before performing a one-tube RT-PCR.

After viral RNA isolation, an equivalent of 1–10 μ L of respiratory specimen was used to reverse transcribe and amplify the NP gene. A one-tube RT-PCR was performed essen-

tially as described by Nijhuis *et al.* (19) using 1.5 mM MgCl₂, 0.4 μ M of primer RS-1 [5'-GGA TTG TTT ATG AAT GCC TAT GGT-3' (Amersham Pharmacia Biotech)] and primer RS-2 (5'-TTC TTC TGC TGT YAA GTC TAR TAC AC-3'). The amount of amplified product was increased further in a nested amplification.

Five microliters of first PCR product is further amplified in a nested amplification, essentially as described by Nijhuis *et al.* (19), using 4.5 mM of MgCl₂ and 0.4 μ M of primer RS-3 (5'-GGA TTC TAC CAT ATA TTG AAC AA-3') and primer RS-4 (5'-CTR TAC TCT CCC ATT ATG CCT AG-3').

Five microliters of nested PCR products were visualized on an ethidium bromide-stained agarose gel using UV illumination. A 100-bp marker was used to control fragment lengths.

Virus preparation. Long-strain RSV was cultured in Hep-2 cells (courtesy of Dr. A.M. van Loon, Department of Virology, University Medical Center, Utrecht, The Netherlands) and 1:1 diluted in sucrose-gelatine solution Z7725a (Laboratory of Vaccine Research, National Institute of Public Health and Environment, Bilthoven, The Netherlands). Titers were determined in Hep-2 cells using the TCID₅₀ method described by Karber (20) (TCID₅₀ 2×10^5). A control antigen was prepared in a similar fashion from uninfected Hep-2 cultures. Virus and control antigen were each prepared as one batch. Virus and control antigen were stored in aliquots at -80°C .

RSV-specific T-cell responses. RSV-specific LPR were performed using a whole blood culture assay as described previously (21, 22).

At three time points, heparinized blood was taken from subjects for whole blood cultures. The first blood sample was taken within 24 h after admission ($t = 1$). In only two of five participating hospitals a blood sample was taken at this time point. The second blood sample was taken 3–4 wk after the initial admission ($t = 2$). The third sample was taken immediately after the second RSV epidemic ($t = 3$). At $t = 3$, RSV-specific LPR, but no cytokine profiles, were determined.

Freshly taken heparinized blood was diluted 1:10 in RPMI 1640 medium (Invitrogen, Carlsbad, CA) and aliquoted (150 μ L) into 96-well culture plates (NUNC A/S, Roskilde, Denmark). Whole blood was infected with RSV at a multiplicity of infection of 0.1–1.0 or control suspension. Cultures were incubated for 6 d at 37°C in 5% CO₂. Forty-eight hours after infection, pooled supernatants were collected for cytokine measurement. Five days after infection, lymphocytes were pulsed with 0.25 μ Ci ³H-thymidine for 18 h and thymidine incorporation was expressed as counts per minute (LPR). Stimulation index was defined as the ratio between LPR in cultures stimulated with RSV and control antigen. A memory response was defined as a stimulation index ≥ 2.0 . All cultures were performed in quadruplicate. All cultures were performed in the same laboratory. Pooled supernatants were kept at -80°C .

Cytokine assays. Cytokines that were measured in supernatants of RSV-stimulated whole blood cultures were IL-4, IL-10, IL-12, and IFN- γ . Concentrations of IL-4, IL-10, and IFN- γ were determined using ELISA kits supplied by the Dutch Laboratory for Blood Transfusion (CLB, Amsterdam, The Netherlands). The detection limit of the assay for IL-4 was 2 pg/mL, for IL-10 2.5 pg/mL, and for IFN- γ 4 pg/mL.

Concentrations of IL-12 were determined using ELISA kits from R & D Systems Europe (Oxford, UK); the detection limit was 7.8 pg/mL. When cytokine values were not detectable, the minimum detectable level was used for statistical analysis.

Statistical analysis. Cytokine production and stimulation indices in RSV-stimulated cultures were logarithmically transformed and expressed as geometric mean and 95% confidence interval (CI). All tests of significance were two sided. Pearson correlation coefficient was used to describe the correlation between LPR at $t = 2$ and $t = 3$. Spearman's correlation coefficient was used to analyze the correlation between RSV-specific LPR or cytokine responses and age. Paired t test was used to analyze differences in (log-transformed) values between different time points. Unpaired t test was used to analyze differences in (log-transformed) values between infants with and without reinfection. All tests of significance were 2-sided. A p value <0.05 was considered statistically significant.

RESULTS

Subject characteristics. The investigated population consisted of 55 patients. Thirty patients (55%) were boys, median age at the time of RSV bronchiolitis was 7 wk. Ten infants (18%) were born prematurely (range 29–36 wk). Ten infants (18%) needed mechanical ventilation. One child had cardiac disease, none of the children had immunodeficiency. None of the prematurely born infants had received RSV prophylaxis. None of the patients received ribavirin or systemic anti-inflammatory agents, including corticosteroids. Patients did not receive inhaled corticosteroids during RSV bronchiolitis. All patients survived.

RSV-specific T-cell responses. A pilot study was performed in samples taken on admission and 3–4 wk after primary RSV infection to determine kinetics of LPR and cytokine responses. At $t = 2$, maximum LPR to RSV were found after 5 d of culture. Maximum IL-10 production was found at 48 h. The pattern and magnitude of IL-10 responses at $t = 1$ and $t = 2$ were comparable and unrelated to stimulation indices. Maximum IFN- γ production at $t = 1$ and $t = 2$ was found after 48 h and 120 h, respectively. IFN- γ and IL-10 responses in control antigen-stimulated cultures were undetectable or relatively low at any time point compared with RSV-stimulated cultures. IL-4 production in RSV-stimulated cultures remained low (<10 pg/mL) and was not higher than IL-4 production in cultures stimulated with control antigen. IL-12 production was not detectable at either $t = 1$ or $t = 2$. We therefore concluded there is no RSV-specific IL-4 or IL-12 production under these culture conditions (data not shown).

At $t = 1$, during the acute phase of RSV bronchiolitis, whole blood cultures were performed in 22 infants. In RSV-stimulated whole blood cultures at $t = 1$, only one patient (4.5%) had a memory response suggesting earlier infection. RSV-induced IFN- γ and IL-10 responses at $t = 1$ were 13 (95% CI: 4–199) and 25.7 (95% CI: 12.5–52.7) pg/mL, respectively. IFN- γ levels were below detection level in 45% and 0% of cases at $t = 1$ and $t = 2$, respectively. IL-10 could be measured in all cases at $t = 1$ and $t = 2$.

At $t = 2$, during the convalescent phase of RSV bronchiolitis, whole blood cultures were performed in 55 infants. Forty-

four patients (80%) had a memory response at $t = 2$, the stimulation index was 5.1 (95% CI: 3.7–8.4). RSV-induced IFN- γ production at $t = 2$ was 60 (95% CI: 48–79) pg/mL, significantly higher than at $t = 1$ ($p < 0.01$). RSV-induced IFN- γ production at $t = 2$ was highly correlated with the RSV-specific LPR at $t = 2$ ($r = 0.80$, $p < 0.001$). RSV-induced IL-10 production at $t = 2$ was 16 (95% CI: 11–23) pg/mL, not significantly different from $t = 1$. At $t = 2$, no significant correlation was found between age and RSV-specific LPR or cytokine responses. Mechanically ventilated infants had higher LPR than nonventilated infants (8.5 and 3.9, respectively, $p = 0.02$), which is in line previous reports (22).

At $t = 3$, after the second winter season, RSV-specific lymphoproliferation was measured in 53 infants. No blood was drawn in two infants (4%). A memory response was found in 20 infants (38%). RSV-specific LPR was significantly lower than after the primary RSV infection (stimulation index = 2.2 versus 5.1, $p < 0.001$). RSV-specific LPR at $t = 2$ and $t = 3$ were highly correlated ($r = 0.40$, $p = 0.001$) (Fig. 1).

Reinfection during second winter season. Eighty-five episodes of respiratory tract symptoms were reported, and reinfection with RSV was diagnosed in 23 infants (42%). In all 23 cases PCR was positive, whereas immunofluorescence was positive in only 9 of 23 cases of RSV infection (39%).

RSV-specific cellular immune responses at $t = 2$ was compared between infants with and without reinfection to assess whether RSV-specific T-cell responses protected against reinfection. No differences in LPR were found between infants with and without reinfection (Fig. 2A). In addition, infants with and without reinfection had comparable RSV-specific IFN- γ (70 versus 54 pg/mL) and IL-10 production (13 versus 16 pg/mL) at $t = 2$.

RSV-specific LPR at $t = 3$ was compared between infants with and without reinfection to assess whether RSV-specific T-cell proliferation was boosted by reinfection. No differences in LPR at

Figure 1. Correlation between RSV-specific lymphoproliferative responses during primary RSV infection and after the subsequent winter season. RSV-specific lymphoproliferative responses [stimulation index (SI)] were measured 3–4 wk after hospitalization for RSV bronchiolitis ($t = 2$) and after the subsequent winter season ($t = 3$). Lymphoproliferative responses were determined by thymidine incorporation in whole blood cultures stimulated with RSV for 5 d. Pearson's correlation coefficient is shown.

$t = 3$ were found between infants with and without reinfection (2.1 and 2.3, respectively) (Fig. 2B). The correlation between LPR at $t = 2$ and $t = 3$ was separately analyzed for infants with and without reinfection. In infants with and without reinfection, the correlation between LPR at $t = 2$ and $t = 3$ was similar ($r = 0.41$, $p < 0.05$ and $r = 0.40$, $p < 0.05$, respectively).

DISCUSSION

The results of this study show that virus-specific T-cell responses induced during primary RSV infection do not protect completely against subsequent reinfection. In addition, reinfection with RSV did not boost RSV-specific LPR.

Reinfections with RSV occur throughout life (23), implying that immunity induced by natural infection provides little long-term protection against reinfection. Naturally induced and exogenously administered RSV-specific antibodies provide incomplete protection, which is of short duration (9, 24–26). To date, sparse

data exist on the protection against reinfection provided by naturally acquired CMI (24, 27). In a follow-up study from the early 1980s, no relationship was found between RSV-specific lymphoproliferative responses induced during primary infection and the risk for subsequent culture-proven reinfection (24). However, it can be doubted whether the sensitivity of viral cultures in this study was suitable to diagnose (usually mild) reinfection (28). Assuming positive PCR for RSV indicates RSV infection, the present study shows that the sensitivity of immunofluorescence for RSV infection in this patient group was only 39%. Thus, PCR, and not culture or immunofluorescence, is required to adequately establish mild reinfection with RSV.

One of the limitations of the present study is that we did not attempt to measure the degree of respiratory disease. Therefore, we cannot exclude that RSV-specific cell-mediated responses decrease the severity of disease in case of reinfection. In addition, in the present study, RSV-specific CMI is represented by virus-specific LPR. Although this is in line with previous studies (22, 27), it is not known what part of CMI is reflected by RSV-specific LPR. The assay measures proliferation in a pool of heterogeneous cells. Therefore, LPR may reflect proliferation of cytotoxic T cells (CTL), cytokine-producing cells, or nonspecific cells that respond to IL-2 produced in the culture system. Cytotoxicity assays measure CTL responses by $CD8^+$ cells and reflect a more accurate effector function of CMI. However, little data are available on CTL responses in infants during RSV infection (29, 30).

To address the role of RSV-specific CTL in protection against infection in the mouse model, vaccination studies with vaccinia virus expressing the M2 protein of RSV were performed. It was shown that protection against RSV infection largely depended on CTL formation (31). CTL responses waned within 45 d after vaccination, which was paralleled by a loss in protection.

In the present study, RSV-specific LPR was induced by primary RSV infection in the majority of infants, which is in line with previous studies (22). Memory was found in one child during the acute phase of disease, whereas 3–4 wk later memory to RSV was found in 80% of the infants. To our surprise, we did not find evidence that reinfection boosted virus-specific CMI, inasmuch as infants with and without reinfection had similar RSV-specific LPR at $t = 2$ and $t = 3$. Moreover, RSV-specific LPR at $t = 2$ and $t = 3$ were highly correlated. Therefore, it can be concluded that RSV-specific LPR induced during primary RSV bronchiolitis partially persists for more than a year and is not boosted by naturally acquired reinfection.

The physiologic role of RSV-specific T cells during reinfection is not well understood. Firstly, RSV-specific T-cell responses did not protect against reinfection. Secondly, virus-specific LPR were induced by primary RSV infection, but not boosted by reinfection. Both findings may be explained by an absence of *in vivo* expansion of RSV-specific T cells during a re-encounter with RSV. It is uncertain, however, why RSV-specific memory T cells are formed during primary infection, but do not proliferate *in vivo* during reinfection.

The immune response during reinfection with RSV is apparently effective, inasmuch as symptoms are usually mild and last for a short period. If RSV-specific T cells do indeed fail to expand *in vivo* during reinfection, it can be questioned whether

Figure 2. RSV-specific lymphoproliferative responses in infants with and without reinfection with RSV. RSV-specific lymphoproliferative responses [stimulation index (SI)] were measured 3–4 wk after hospitalization for RSV bronchiolitis ($t = 2$) (A) and after the second winter season ($t = 3$) (B) in infants with and without reinfection with RSV. Lymphoproliferative responses were determined by thymidine incorporation in whole blood cultures stimulated with RSV for 5 d. Geometric means are shown.

T-cell memory plays a major role in the immune response during reinfection. B-cell memory may be more important during reinfection. This is supported by a prominent rise in neutralizing antibodies observed after reinfection with RSV (8). In addition, nonadaptive immunity could have a function in the elimination of RSV during reinfection. Natural killer (NK) cells are important in the defense against viruses because they are capable of providing cytotoxicity and producing cytokines, including IFN- γ (32). Although no data exist on the role of NK cells during RSV infection in humans, this cell type has been implicated in the immune response during RSV infection in mice (33, 34). In addition, type I interferons (IFN- α/β) could play a role in the antiviral immune response. These cytokines with direct antiviral properties are produced in high amounts by airway epithelium upon infection with RSV (35) and they are found in the airways of RSV-infected infants (36).

An important aim in the development of a vaccine against viruses is the induction of virus-specific memory. Subsequent encounter with the virus should result in expansion of at least part the pool of memory T cells. The present study clearly shows that RSV-specific T cells do not necessarily expand during reinfection. If a future RSV vaccine for humans induces RSV-specific T cells, which lack the potential to expand *in vivo* upon natural infection, this could have implications for the effectiveness of the vaccine. Limited protection by memory T cells would be expected, and the effect of the vaccine would then largely depend on the formation of B-cell memory. It remains to be seen whether B-cell memory is sufficient for protection against reinfection.

In conclusion, the present study shows that RSV-specific T-cell responses do not protect against reinfection. Moreover, reinfection does not boost RSV-specific LPR. Together, these findings suggest that RSV-specific T cells do not expand *in vivo* upon reinfection with RSV, which could bear relevance for the development of an effective vaccine.

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