Correlation between Torque Tenovirus Infection and Serum Levels of Eosinophil Cationic Protein in Children Hospitalized for Acute Respiratory Diseases

Fabrizio Maggi, Massimo Piñero, Elena Tempestini, Lotizia Lanini, Emanuela De Marco, Claudia Formai, Elisabetta Andreoli, Silvano Presciutti, Maria Linda Vatteroni, Mauro Pistelli, Vincenzo Ragazzo, Pierantonio Macchia, Angelo Pietrobelli, Attilio Bone, and Mauro Bendinelli

Virology Section and Retrovirus Center, Department of Experimental Pathology, and Department of Pediatrics, University of Pisa, Pisa, and Department of Pediatrics, University of Verona, Verona, Italy

Children with bronchopneumonia have considerably higher Torque tenovirus (TTV) loads than do children with milder acute respiratory diseases (ARDs). Moreover, in children with ARDs, high TTV loads correlate with low percentages of circulating CD3+ and CD4+ T cells and with elevated percentages of B cells, suggesting that TTV might be immunomodulatory. Here, we show that, in children with ARDs, the presence of TTV and TTV load correlates with concentrations of serum eosinophil cationic protein. The possible mechanisms whereby TTV infection might lead to augmented activity of eosinophils and the implications for pathogenesis are discussed.

In children, acute respiratory diseases (ARDs) are a frequent cause of hospitalization. Numerous common respiratory viruses are known to be responsible for ARDs that require hospitalization. Nonetheless, a significant proportion of the cases of ARD occurring in this age group, as well as in other age groups, remain etiologically unresolved [1].

Torque tenovirus (TTV) is a single-stranded DNA virus of ~3800 nt classified in the new genus Anellovirus [2]. TTV is genetically highly heterogeneous and has been subdivided into 5 genogroups and >40 genotypes [3]. TTV produces long-lasting (possibly life-long) viremia in ~80% of apparently healthy individuals of all ages throughout the world [4]. Recently, we demonstrated that, in children hospitalized for ARD, the respiratory tract is a site of primary TTV infection and continual TTV replication [5]. Of interest, although no evidence was obtained that TTV might be a direct cause of ARD, average TTV loads were considerably higher in children with bronchopneumonia (BP) than in children with milder illnesses, regardless of the presence of common respiratory viruses. In such children, TTV loads were also inversely correlated with the percentages of circulating CD3+ and CD4+ T-cells and directly correlated with the percentage of B cells, suggesting that TTV might have immunomodulatory effects [6].

The eosinophil cationic protein (ECP) is produced in large amounts by both mature and immature eosinophils and is released in large amounts when these cells undergo activation and degranulation [7]. Concentrations of ECP in the circulation may be elevated by inflammatory conditions of the lower respiratory tract, including viral infection, unexplained cough, and asthma [8]. Furthermore, inflammation of eosinophils is a hallmark of the pathogenesis of asthma and wheezing manifestations in general [9], and concentrations of serum ECP (s-ECP) have been proposed to be a predictive marker for the development of asthma during childhood [8, 10], as well as a tool for defining disease type and monitoring disease severity and responsiveness to therapies [8]. In the present study, we measured the concentrations of s-ECP in 139 children with ARDs and correlated them with the presence of TTV and with TTV loads. The results provide a further element arguing in favor of a significant role of TTV in the etiopathogenesis of respiratory disease in young children.

Patients, materials, and methods. The patients were 139 children (85 boys and 54 girls) with ARDs, aged 1–24 months (mean ± SD, 8.0 ± 5.9 months), admitted to the Department of Pediatrics, University Hospital, Pisa, Italy, from November 1999 to April 2003. All the children had been born at a gestational age of >36 weeks and were negative for hepatitis B and C viruses and HIV-1 and -2 infections. None of the children had any recognized congenital or genetic syndrome or allergic disease, such as atopic dermatitis, and none had received blood transfusions or antiviral drugs. During hospitalization, none of the children required intensive care or mechanical ventilation. When the children were discharged, the following diagnoses were defined: laryngotracheobronchitis (n = 14), bronchiolitis (n = 88), and BP (n = 37).

Informed consent was obtained from the parents of all children who provided samples. Contemporaneous blood and nasal
swabs were obtained on the morning of the first day of hospital admission. To avoid influencing concentrations of s-ECP during the coagulation process, serum was obtained in a strictly standardized manner. In brief, 2.5 mL of blood was kept at room temperature for 1 h and subsequently was centrifuged for 10 min at 1300 g at 4°C. Serum was then collected, centrifuged again to remove additional eosinophils that may have remained in the samples, and finally stored at −20°C until use. Concentrations of s-ECP were determined by use of a commercial radioimmunoassay (UniCAP 100; Pharmacia and Upjohn). All s-ECP measurements were done in duplicate. The intra-assay and interassay coefficients of variation were <3%, and the limit of detection was 2 ng/μL. When test results were negative, the value of the lower limit of detection of the assay was arbitrarily used in calculations. Peripheral blood eosinophils were counted by use of an automated analyzer (H1 System; Technicon Instruments). Total IgE was measured nephelometrically (Behring). In limited numbers of blood samples, the concentrations of interleukin (IL)–1, IL-2, IL-4–6, IL-8, IL-10, IL-12, and IL-13 and of total IgA, IgG, and IgM were measured by use of commercial immunological and nephelometric assays, respectively.

Nasal swabs and aliquots of blood were transferred to the Clinical Virology Laboratory of the Department of Experimental Pathology (University of Pisa), where they were immediately processed. Nasal swabs were vortexed in 0.5 mL of sterile saline, to achieve a homogeneous suspension of nasal secretions, and were tested for common respiratory viruses by use of direct immunofluorescence assay (adenovirus, cytomegalovirus, influenza viruses A and B, parainfluenza types 1–3, and respiratory syncytial virus [RSV]), EIA (influenza viruses A and B and RSV), rapid culture in shell vials (all viruses mentioned above), or polymerase chain reaction (PCR) (rhinoviruses and metapneumovirus). In accordance with the clinical indications, the swabs were not examined for the presence of bacterial pathogens. Plasma was obtained by spinning down blood collected in sodium citrate and was stored at −80°C until use.

Viral DNA was extracted from 200 μL of plasma by use of the QIAamp DNA Mini kit (QIAgen). The presence of TTV and TTV load were determined by use of a universal TaqMan real-time PCR, exactly as described elsewhere [5, 6]. Because this PCR is targeted to a highly conserved segment of the noncoding region of the viral genome, it has the potential to sensitively and specifically detect all known TTV genotypes deposited in GenBank. The procedures used for quantification of copy numbers and evaluation of specificity, sensitivity, intr assay and interassay precision, and reproducibility of the assay have been described elsewhere [5, 6, 11].

Selected samples found to be TTV positive by the universal PCR were amplified by 5 group-specific PCR protocols [5, 6]. All samples were tested at least in duplicate. Specificity and sensitivity of each group-specific assay were measured as described elsewhere [5, 6].

Pearson’s χ² test or Fisher’s exact test was applied to evaluate the heterogeneity of contingency tables. Differences between means were evaluated by use of the 2-tailed Student’s t test. Associations between variables were determined by use of Pearson’s correlation coefficient. A receiver-operating characteristic (ROC) curve was performed to calculate the optimal cutoff for concentrations of s-ECP. Multivariate logistic regression analysis was used to calculate the adjusted odds ratio (OR) for independent variables.

Results. TTV was detected in 112 (80%) of 139 children tested, at plasma loads of 3.1–8.2 log₁₀ copies/mL (table 1), similar to virus prevalence and loads previously found in both healthy and ill adults in the same geographic area [11]. At admission, differences between the TTV-positive and -negative children, with regard to all major clinical variables (e.g., mean duration of respiratory symptoms, mean respiratory rate, mean oxygen saturation, and mean total serum IgE), were not statistically significant. The geometric mean peripheral blood eosinophil count was 29.3 cells/μL (95% confidence interval [CI], 23.7–36.3 cells/μL) in the TTV-positive children and 23.3 cells/μL (95% CI, 18.1–31.0 cells/μL) in the TTV-negative children, but the difference was not statistically significant. In spite of considerable individual variability, concentrations of s-ECP were significantly higher in the TTV-positive children (geometric mean, 7.8 ng/mL [95% CI, 6.6–9.2 ng/mL]) than in the TTV-negative children (geometric mean, 5.1 ng/mL [95% CI, 3.9–6.6 ng/mL]) (P = .019) (table 1). Furthermore, a direct correlation was observed between TTV loads and concentrations of s-ECP (r = 0.276; P = .009).

The threshold above which concentrations of s-ECP should be considered to be elevated has proved to be somewhat difficult to establish with certainty [7], but several reports have indicated that the threshold is 16 ng/mL [9, 12]. As shown in table 1,

<table>
<thead>
<tr>
<th>TTV load</th>
<th>Total no. of children in the group</th>
<th>No. (%) of children with s-ECP &gt;16 ng/mL</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undetectable</td>
<td>27</td>
<td>1 (4)</td>
<td>...</td>
</tr>
<tr>
<td>Detectable, log₁₀ copies/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0–3.9</td>
<td>21</td>
<td>4 (19)</td>
<td>NS</td>
</tr>
<tr>
<td>4.0–4.9</td>
<td>30</td>
<td>3 (10)</td>
<td>NS</td>
</tr>
<tr>
<td>5.0–5.9</td>
<td>27</td>
<td>4 (15)</td>
<td>NS</td>
</tr>
<tr>
<td>≥6.0</td>
<td>34</td>
<td>11 (32)</td>
<td>.008</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td>22 (20)</td>
<td>.047</td>
</tr>
</tbody>
</table>

NOTE. NS, not significant.

² Difference from undetectable TTV (Fisher’s exact test).

Table 1. Torque tenovirus (TTV) loads and presence of high concentrations of serum eosinophil cationic protein (s-ECP) in the study children.
the proportion of children with concentrations of s-ECP >16 ng/mL was significantly higher in the TTV-positive than in the TTV-negative children. Furthermore, the difference was evident in all the children, regardless of TTV load, although it reached statistical significance only in the children with plasma TTV loads ≥6.0 log_{10} copies/mL. We also applied an ROC curve analysis to the concentrations of s-ECP in relation to TTV loads (<6.0 and ≥6.0 log_{10} copies/mL). The most discriminating value of s-ECP was 15.4 ng/mL, thus supporting the threshold value of 16 ng/mL selected. Table 2 shows the results of a logistic regression analysis that included concentration of s-ECP >16 ng/mL as a dependent variable and 7 independent variables, all of which were measured in the same samples used for detection of TTV. Of interest, of the 3 variables that were found to be associated with concentrations of s-ECP >16 ng/mL with P < .05 (overall P value of the model, 0.001), the variable TTV load ≥6.0 log_{10} copies/mL showed the highest OR.

To ascertain whether the presence of concentrations of s-ECP >16 ng/mL could be preferentially associated with infection by multiple and/or specific genetic forms of TTV, selected TTV-positive children who had concentrations of s-ECP >16 ng/mL (n = 16) or <16 ng/mL (n = 15) were characterized for the TTV genogroups they harbored. In line with previous findings [5, 6], mixed TTV infections were a frequent occurrence (6 of the 31 children examined for mixed TTV infections harbored as many as 4 TTV genogroups), and genogroups 1 and 3 were highly predominant. More important, no major differences between the 2 groups of children were detected, with regard to either the number or the identity of the TTV genogroups carried, thus excluding the possibility that these variables were important determinants in the occurrence of elevated concentrations of s-ECP. As determined in subsets of 25–39 children, mean concentrations of IL-1, IL-2, IL-4–6, IL-8, IL-10, IL-12, and IL-13 and of total IgA, IgG, and IgM were also similar in TTV-positive and -negative children. The mean duration of hospital stay was also similar for the 2 groups of children.

Discussion. The mechanisms behind the strong association observed between TTV infection and concentrations of s-ECP remain unclear. That increased inflammation may lead to augmented replication of TTV seems unlikely in view of the fact that no positive correlation was observed between TTV loads and levels of the inflammatory cytokines (IL-1, IL-6, and IL-12) in plasma (data not shown). Eosinophils are, by far, the major source of ECP in serum, as well as in nasopharyngeal fluids [7, 8]; thus, increased numbers of eosinophils might be a factor in the observed correlation. Peripheral blood eosinophil counts were only moderately increased in the TTV-positive children, but this result does not exclude the possibility that there is augmented recruitment of eosinophils in the airways. Elevated concentrations of s-ECP have also been described in RSV-infected children with bronchiolitis [13, 14], although not in the present study, and have been associated with augmented eosinophilia in the airways [15]. Degranulation of eosinophils and consequent increased secretion of their granule proteins are known to be induced by a wide range of stimuli, including IgG, IgA, IgE, and cytokines (e.g., IL-3 and IL-5) [7]. Thus, an alternative possibility is that TTV may increase eosinophil degranulation either by direct interaction with these cells or by induced modifications in the concentrations of factors that incite this event. It should be noted, however, that measuring levels of circulating immunoglobulin and several cytokines, including IL-5, in some of our children provided no clues in this direction.

ECP is a multifunctional glycoprotein; its recognized activities include cytostatic and cytotoxic effects, inhibition of lymphocyte activity, stimulation of release of histamine and other reactive molecules by mast cells and basophils, and stimulation of airway mucus secretion [7]. Moreover, ECP is just one of the many mediators released by activated eosinophils that may produce inflammation, epithelial cell injury, and recruitment of additional inflammatory cells [8]. Thus, it is possible that ECP and other eosinophil-produced mediators participate in damage to airways and contribute to the severity of ARDs. Finally, it is worth mentioning that other studies have shown that, in young children, high concentrations of s-ECP predict an increased likelihood of developing airway hyperreactivity, wheezing illnesses, and asthma later in life [8, 13, 15]. Therefore, the findings of the present study raise the interesting possibility that TTV infection represents a heretofore unrecognized inducer or, more likely, facilitator of the pathophysiological

### Table 2. Odds ratios (ORs) for having increased concentrations of serum eosinophil cationic protein >16 ng/mL for 7 independent variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, months</td>
<td>1.1 (1.0–1.2)</td>
<td>.002</td>
</tr>
<tr>
<td>Peripheral blood eosinophil count, cells/μL</td>
<td>2.7 (1.0–7.0)</td>
<td>.04</td>
</tr>
<tr>
<td>TTV load ≥6 log_{10} copies/mL</td>
<td>3.0 (1.1–8.4)</td>
<td>.03</td>
</tr>
<tr>
<td>X-ray diagnosis of BP^a</td>
<td>0.5 (0.1–2.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Total serum IgE^a</td>
<td>1.3 (0.5–3.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Presence of RSV^a</td>
<td>0.5 (0.1–1.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Presence of any common respiratory viruses^a,b</td>
<td>1.1 (0.4–3.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NOTE. BP: bronchopneumonia; CI: confidence interval; NS, not significant; RSV, respiratory syncytial virus; TTV, Torque tenovirus.

^a This variable was removed from the last model, since its P value was not statistically significant (> .05 at the 95% confidence level).

^b Fifty-one children (37%) were positive for RSV.

^c Eighty-five children (60%) were positive for any common respiratory viruses.

Detection of metapneumovirus was performed for only 26 children, with 8 positive results, 6 of which were for children who were positive for other common respiratory viruses; those results were not included in the calculations.
processes of these common afflictions. That susceptibility to wheezing after exposure to eliciting stimuli could be related to factors already in place has been suggested before [15]. Because TTV infection is so pervasive, it might represent one such factor and might act by priming eosinophils toward an accelerated or enhanced release of ECP and other effector molecules. Further studies in this area are clearly warranted.

References