T – Helper2 / T – Helper1 Imbalance in Respiratory Syncytial Virus Bronchiolitis in Relation To Disease Severity and Outcome

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The present study included 80 Egyptians infants with acute bronchiolitis; age ranged from 2 to 22 month and consisted of 60 males and 20 females. In addition, 30 apparently healthy infants of matched age and sex were also included as controls. Besides taking history, thorough clinical examination and routine laboratorial investigations, arterial oxygen saturation, RSV tissue culture of nasopharyngeal lavage, indirect immunofluorescence for serum IgM against RSV, serum IL-4 and IFN-γ were done. Results obtained showed that viral bronchiolitis is predominant in male around 6 month of age and in winter months. RSV was identified in 72.5% of cases. Level of serum IL-4 was significantly higher in RSV positive patients than either RSV negative patients or controls ($P<0.001$). Furthermore, in RSV positive patients, IL-4 level was significantly higher in severe and fatal cases than in milder and surviving cases ($P<0.001$). Serum IFN-γ showed no significant difference between RSV positive and RSV negative patients In RSV positive cases it was significantly lower in more severe and fatal cases than milder and living ones ($P<0.05$). The ratio IL-4/IFN-γ was significantly higher in RSV positive than RSV negative and controls ($P<0.001$ and $P<0.05$ respectively). Absolute eosinophilic count (AEC) and percent of oxygen saturation (SaO2 %) showed significantly lower values in RSV positive patients than RSV negative ($P<0.001$), but the latter showed significantly higher AEC than controls ($P<0.05$). In RSV positive group, both parameters were significantly lower in more severe and fatal cases than milder and living ones ($P<0.001$). In RSV positive group, significant positive correlations were found between disease severity and either serum IL-4 or IL-4/IFN-γ ratio ($r=0.789$ and $r=0.823$, $P<0.001$ respectively) but disease severity was inversely correlated with either AEC or SaO2 % ($r=-0.962$ and $r=-0.828$, $P<0.001$ respectively). RSV was identified as the major etiologic virus of bronchiolitis in young infants. Viral tissue cultures and indirect immunofluorescence are of equally diagnostic value but both may be needed. Most of the severe and fatal cases are in RSV positive group. Th2/Th1 imbalance reflected on IL-4/IFN-γ ratio is more deranged in RSV bronchiolitis with Th2 predominance especially in more severe and fatal cases. These conclusions may be a rationale for the implementation of antiviral drugs in severe cases of bronchiolitis, however, further studies are needed to demonstrate the effect of antiviral drugs on cytokines levels during therapy.

Respiratory syncytial virus (RSV) is a single stranded negative sense RNA virus in the paramyxovirus family that is a major cause of morbidity and life threatening lower respiratory tract disease (LRTD) in infants and young children worldwide (Tripp et al., 2005). Acute respiratory failure associated with severe bronchospasm, hypoxia and carbon dioxide retention necessitate hospitalization of a fraction of 1-2% of RSV-infected children (Simoes, 1999). Viral bronchiolitis is an important manifestation of LRTD, particularly among infants (Shay et al., 2001). It occurs during the first two years of life, with a peak incidence at six months of age. It imposes a substantial economic burden on developing countries (Roopal et al., 2001).

RSV infection may be associated with short- and long- term morbidity including complications such as recurrent wheezing, reactive airway disease and pulmonary function abnormalities (Openshaw et al., 2003).

There is convincing evidence that Th1 and Th2- type cytokine patterns, determine the type of immune response to RSV infection. The spectrum of cytokine expression affects control mechanisms involved in the regulation of RSV disease pathogenesis and chronicity.
In addition, balance between virus elimination and disease pathogenesis and severity is also affected (Welliver, 2003; Durbin & Durbin, 2004; Tripp, 2004). This study aimed to delineate the diagnostic significance of viral tissue culture and RSV serum IgM immuno-fluorescence for RSV LRT infections. Another objective was to study the association of Th2/Th1 imbalance and disease severity.

**Subjects and Methods**

The present study included 80 infants who attended to Pediatric Department during January 2006 to January 2007. Study children presented with symptoms and signs suggestive of acute viral bronchiolitis; tachypnea, wheezes with prolonged expiratory phase and crackles on auscultation (Simoes, 1999) and overinflated lungs both clinically and by chest x-rays. Their ages ranged from 2 to 22 months (60 males and 20 females). Prior to enrolling infants in this study, consent was obtained from their parents. Twenty eight patients were enrolled infants of matched age and sex were also enrolled in the study as controls. They were coming for fitness and assessment of haemostatic status before minor procedures such as (circumcision); their parents gave consent to participate in the study. This study was approved by the Medical Ethical Committee of Assiut University Hospitals.

**Collection of Specimens**

Nasopharyngeal aspirates were collected from all patients within 24 hours of admission by irrigation and suction of both nostrils with one milliliter of saline using a sterile blunt tipped disposable catheter and syringe. Specimens were increased to 3 ml with Hank's balanced salt solution (HBSS) containing a protein stabilizer (2% fetal calf serum), 100 units penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 0.25 µg/ml amphotericin. Specimens were then placed on ice and transported to the laboratory within 2 hours after collection. Samples were processed directly or were stored at -70°C.

Five ml of blood were collected from each patient and control. Two ml were added in a tube containing EDTA (ethylene diamine tetra-acetic acid) for complete blood count and absolute eosinophilic count using Cell Dyon 3500 hematologic analyzer. The remaining 3 ml were added in a plain tube, and centrifuged at 3,000 rpm for 10 min. Serum was then divided into aliquot and stored at -70°C for further analysis.

**Arterial Oxygen Saturation**

Arterial oxygen saturation (SaO₂ %) was done using the non-invasive percutaneous pulse oximeter (Model 520 A, NOVAMETRIX, Medical Systems Inc., USA).

**Viral Culture and Direct Immunofluorescence**

Nasopharyngeal aspirate samples (0.4 ml) were inoculated into two tissue culture flasks containing Vero cells monolayers, in duplicates. Cultures were observed daily for 10 days for cytopathic effect (CPE).

**Viral Serology**

Serum RSV specific IgM antibodies were detected by using a commercially available indirect immuno-fluorescence kit (VIRCELL Diagnostic, Spain).

**Cytokine Assay**

Serum IL-4 and IFN-γ were measured using enzyme linked immunosorbent assay (ELISA) kits catalogue numbers KAP1281 and KAP1231, respectively supplied by Biosource, Europe S.A (Belgium). The detection limit for IL-4 was 1.3 pg/ml and for IFN-γ was 0.03 pg/ml. As a measure of the balance between Th2 / Th1 immune response, the ratio of IL-4 / IFN-γ was calculated for each subject Legg et al., (2003).
Statistical Analysis

Analysis of demographic data expressed as numbers and percentages was done using Chi square. Laboratory data were expressed as mean ± standard deviation. Significance was calculated using the Mann Whitney U test for the wide variable values and paired-t test for homogenous values. P values <0.05 were considered significant. Regression analyses were performed using Spearman’s rank correlation coefficient analysis.

Results

Out of the 80 patients, 12 infant died all of them presented with severe bronchospasm and three infant were having underlying congenital heart disease. Regarding distribution of all patients with bronchiolitis, it was significantly higher for in males than females, for full term than infants with preterm delivery, for those aged >6 months, in winter than non winter months and for those with negative family history of wheeze (allergic disease and chronic respiratory air way diseases) than those with positive history (Table 1). Similarly, RSV positive bronchiolitis cases were identified more frequently during winter season (Table 1).

Table 1. Demographic and clinical data of patients with bronchiolitis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number of cases</th>
<th>%</th>
<th>*P&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (m) ≤ 6 / &gt; 6</td>
<td>26 / 54</td>
<td>32.5 / 67.5</td>
<td>0.05</td>
</tr>
<tr>
<td>Sex (M / F)</td>
<td>60 / 20</td>
<td>75 / 25</td>
<td>0.01</td>
</tr>
<tr>
<td>Season (winter / non-winter)</td>
<td>64 / 16</td>
<td>80 / 20</td>
<td>0.001</td>
</tr>
<tr>
<td>Passive smoking (positive/ negative)</td>
<td>32 / 48</td>
<td>40 / 60</td>
<td>NS</td>
</tr>
<tr>
<td>Severity (mild &amp; moderate / severe)</td>
<td>52 / 28</td>
<td>65 / 35</td>
<td>0.05</td>
</tr>
<tr>
<td>Frequency of attacks (≤ 2 attacks / &gt; 2 attacks)</td>
<td>22 / 58</td>
<td>27.5 / 72.5</td>
<td>0.05</td>
</tr>
<tr>
<td>Family history of wheeze (positive / negative)</td>
<td>20 / 60</td>
<td>25 / 75</td>
<td>0.01</td>
</tr>
<tr>
<td>Gestational age (preterm / full term)</td>
<td>18 / 62</td>
<td>22.5 / 77.5</td>
<td>0.01</td>
</tr>
<tr>
<td>RSV positive patients (58/80)</td>
<td>58</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Number of attacks (≤ 2 attacks / &gt; 2 attacks)</td>
<td>28 / 30</td>
<td>48.27 / 51.73</td>
<td>NS</td>
</tr>
<tr>
<td>Severity (mild &amp; moderate / severe)</td>
<td>30/28</td>
<td>51.73 / 48.27</td>
<td>NS</td>
</tr>
<tr>
<td>Season (winter / non-winter)</td>
<td>50 / 8</td>
<td>86.2 / 13.8</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*p<0.05 is significant. NS= not significant

SaO₂% was significantly different (P<0.001) in patients with RSV infection (77±16) than in patients with bronchiolitis and negative for RSV (91.9±3.6). Absolute Eosinophilic (AEC) count was significantly higher (P<0.01 and P<0.05) higher in RSV negative infants (222.3±50.5) than in RSV positive (103.5±98.6) and in controls (166.7±115.9). Furthermore, both AEC and SaO₂% values were significantly lower (P<0.001) in severe RSV positive patients than milder group and in those who died than improving group (Table 3).

The main causative organism of viral bronchiolitis occurring in study infants was RSV. The virus was identified by culture and fluorescein-labelled anti-mouse antibodies in nasopharyngeal aspirates of 72.5% of infants (n=58/80) with bronchiolitis. In addition, serum IgM antibodies to RSV was detected 72.5% (n=58/80) of infants with bronchiolitis.

Serum IL-4 level was significantly higher in infants with bronchiolitis and positive for RSV as compared to either infants with bronchiolitis but not RSV positive or controls (P<0.001). However, levels of serum IL-4...
were not significantly different between infants with bronchiolitis and negative for RSV and controls (Table 2). On the other hand, levels of IFN-γ observed in patients with bronchiolitis with or without RSV infection were significantly ($P<0.001$) higher than in control group (Table 2). No significant difference was observed between patients positive and negative for RSV. The ratio IL-4/IFN-γ was significantly higher ($P<0.001$ and $P<0.05$) in infants positive for RSV than in infants negative for RSV and controls, respectively. Moreover, IL-4 / IFN-γ ratio was lower ($P<0.001$) in the RSV negative group than controls (Table 2).

In addition, we compared RSV positive group (n=58) with respect to frequency of attacks, disease severity and outcome (Table 3). No significant difference in all measured parameters in relation to the attack frequency. Regarding disease severity and outcome, serum IL-4 level was significantly higher ($P<0.001$) in severe group than milder and in fatal outcome group than in surviving cases. Serum IFN-γ levels were significantly lower in severe and in fatal cases ($P<0.05$).

### Table 2. Serum IL-4, IFN-γ, IL-4 / IFN-γ ratio, A.E.C. and SaO₂% in patients and control groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-4 (pg / ml)</th>
<th>IFN-γ pg / ml</th>
<th>IL-4 / IFN-γ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I- RSV positive Patients</td>
<td>32.01 ± 22.08$^a$</td>
<td>20.95 ± 11.92$^b$</td>
<td>2.18 ± 1.78$^{cd}$</td>
</tr>
<tr>
<td>n=58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II- RSV negative patients</td>
<td>4.40 ± 2.08</td>
<td>15.99 ± 8.38$^b$</td>
<td>0.32 ± 0.12$^a$</td>
</tr>
<tr>
<td>n=22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III- Controls (n=30)</td>
<td>3.39 ± 1.88</td>
<td>4.06 ± 2.17</td>
<td>0.89 ± 0.42</td>
</tr>
</tbody>
</table>

$^aP<0.001$ RSV positive as compared to RSV negative and controls
$^bP<0.001$ RSV positive and RSV negative as compared to controls
$^cP<0.001$ RSV positive as compared to RSV negative.
$^dP<0.05$ RSV positive as compared to controls
$^eP<0.001$ RSV negative as compared to controls

### Table 3. Cytokine levels in RSV positive patients in relation to frequency of attacks, severity and outcome.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Frequency</th>
<th>Severity</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ 2 attacks</td>
<td>&gt; 2 attacks</td>
<td>Mild &amp; moderate</td>
</tr>
<tr>
<td></td>
<td>(n = 28)</td>
<td>(n=30)</td>
<td>(n=30)</td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>29.69 ± 20.63</td>
<td>34.18 ± 23.86</td>
<td>15.7 ± 7.7</td>
</tr>
<tr>
<td>IL-4/IFN-γ ratio</td>
<td>2.24 ± 2.06</td>
<td>2.12 ± 1.55</td>
<td>0.9 ± 1.15</td>
</tr>
<tr>
<td>AEC*</td>
<td>116.43± 108.31</td>
<td>91.4 ± 90.65</td>
<td>179.69 ± 65.15</td>
</tr>
<tr>
<td>SaO₂%**</td>
<td>77.50± 19.96</td>
<td>77.47 ± 11.97</td>
<td>89.31 ± 7.19</td>
</tr>
</tbody>
</table>

$^a$AEC = absolute eosinophilic count; $^b$SaO₂% = Percent of oxygen saturation
$^cP<0.001$ Severe as compared to mild to moderate infection and in fatal as compared to surviving cases (IL-4)
$^dP<0.05$ Severe as compared to mild to moderate infection and surviving as compared to fatal outcome (IFN-γ)
$^eP<0.001$ Severe as compared to mild to moderate cases and fatal cases as compared to surviving cases (AEC and SaO₂%)

A significant positive correlation ($r =0.789$, $P<0.001$ and $r=0.823$, $P<0.001$) was denoted between disease severity and either serum IL-4 or IL-4/IFN-γ ratio, respectively, in RSV positive group (Figures 1 and 2). On the other hand, a significant negative correlation ($r=-0.962$, $P<0.001$ and $r=-0.828$, $P<0.001$) was observed between disease severity and either of AEC or SaO₂ % in RSV negative group, respectively (Figure 3 and 4).
Figure 1. Correlation between disease severity and IL-4 in RSV positive group

Figure 2. Correlation between disease severity and IL-4/IFN-γ in RSV positive group

Figure 3. Correlation between disease severity and AEC in RSV positive group
Discussion

Viral bronchiolitis is a cause of lower respiratory tract illness (LRTI) particularly among infants (Shay et al., 2001). It is the most common cause of hospitalization imposing an economic burden on developing countries (Roopal et al., 2001). The present study showed that RSV is the most frequent causative agent of bronchiolitis in Egyptian infants, occurring mainly during winter season. Our results corroborate previous results that as many as 50-90% of infants hospitalized during winter with bronchiolitis are infected with RSV (Hall, 2001). Also, Openshaw et al. (2003) stated that RSV is the most important cause of serious lower respiratory tract disease in young children. Furthermore, Zeinab et al., (2002) isolated RSV in 60.5% of infants with bronchiolitis.

The present study showed that AEC and SaO₂% were significantly lower in RSV positive patients than RSV negative. In addition, AEC and SaO₂% were also significantly lower in more severe RSV positive and those with poor outcome than mild to moderate RSV positive patients and those who improved. Our results are in line with Pinto et al. (2006) and Zewang & Harrod, (2006) who reported that RSV infection mostly cause severe lower respiratory disease and lead to esinopenia. Furthermore, they documented that severe RSV bronchiolitis is associated with increased plasma cortisol that decrease AEC which are very sensitive cells to the effect of glucocorticoids. To the best of our knowledge, the observed significant negative correlations between the severity of RSV bronchiolitis and either AEC or SaO₂% in our study support such data.

In line with our data, Pinto and his colleagues (2006) found an inverse correlation between Th1/Th2 ratio and severity of RSV bronchiolitis or in other words a positive correlation between Th2/Th1 (IL-4/IFN-γ) and severity of the disease. This observation could be explained by the fact that increased severity causes decreased oxygen saturation that induces stress stimulating cortisol over production. The increased cortisol inhibits Th1 immune response and decrease serum IFN-γ (Pinto et al., 2006). Furthermore, Zewang & Harrod (2006) stated that RSV has a unique characteristic that enhances Th2 and IL-4 production and inhibits or causes less stimulation of Th1 and IFN-γ leading to an increase in IL-4/IFN-γ ratio in RSV infection especially in severe cases. Legg et al. (2003) attributed this marked Th2/Th1 imbalance during RSV bronchiolitis to the less efficient
viral clearance but not to the difference in initial viral load. Also, they stated that high IL-4 in severe cases delays viral clearance and inhibits interferon-γ production. So, the noticed significantly lower serum IFN-γ in severe and fatal RSV positive patients than mild to moderate ones supports this fact. Also, Roman and his colleagues (1997), found significantly increased IL-4 / IFN-γ ratio in infants with RSV bronchiolitis compared with healthy controls. Similarly, Aberle et al. (1999) identified lower levels of IFN-γ in infants with severe RSV bronchiolitis than in those with milder course suggesting a preference toward the Th2 response in relation to severity of the disease. Furthermore, Bendelja et al. (2000), observed an increased ratio of intracellular IL-4/IFN-γ in the peripheral blood mononuclear cells obtained from RSV-infected infants compared with healthy controls. In contrast to these observations and to our results; Brandenburg et al. (2000), Garofalo et al. (2001) and Van Banten et al. (2003) noticed no difference in interleukin levels that differentiate between Th2 and Th1 immune response in RSV patients regardless of the disease severity. These different results may be due to a combination of factors relating to study design, such as different types and treatment of samples and timing of sampling in relation to the onset of viral infection. Difference in virus load in different study groups, age and family history of atopy may also explain the spectra of results (Psarras et al., 2004).

Viral bronchiolitis is a disease of winter months affecting mainly infants around six months of age. Respiratory syncytial virus is the major etiologic factor causing more severe disease than non- RSV bronchiolitis and carries a bad outcome. Viral tissue culture and indirect immunofluorescence are valuable for diagnosis of RSV. The ratio IL-4/IFN-γ is altered in RSV positive bronchiolitis cases with bias toward IL-4 response, especially in severe and cases with a fatal outcome. Data obtained in this study suggest that antiviral therapy should be implemented in severe cases of bronchiolitis, however, further studies are needed to address cell mediated immunity and diverse cytokines responses in RSV patients.

References
Th2 / Th1 Imbalance in RSV Bronchiolitis in Relation To Disease Severity and Outcome


