Abundant IFN-γ production by local T cells in respiratory syncytial virus-induced eosinophilic lung disease

Lindsay C. Spender,† Tracy Hussell and Peter J. M. Openshaw

Respiratory Medicine, National Heart and Lung Institute, Imperial College School of Medicine at St Mary’s, Norfolk Place, London W2 1PG, UK

Respiratory syncytial virus (RSV) is a frequent cause of severe lung disease in young children. Primed T cells are required for virus clearance, but are causally implicated in the enhanced pathology seen following RSV infection of some infants and experimental animals vaccinated against the virus. In BALB/c mice, vaccination with recombinant vaccinia virus expressing the viral attachment protein (G) leads to pulmonary eosinophilia during subsequent infection, which indirect evidence suggests may be due to CD4+ Th2 cells. The production of IFN-γ, IL-2, -4, -5 and -10 cytokine mRNA by RT–PCR and intracellular cytokines by flow cytometry following RSV challenge of vaccinated mice were therefore compared. Lung eosinophila was associated with enhanced local recruitment of CD4+ cells in G sensitized mice, while CD8+ cells dominated in mice vaccinated with the viral fusion protein (F) or second matrix protein (M2). Lung eosinophila was also associated with a localized reduction in IFN-γ and increased IL-4 and IL-5 mRNA transcription as well as elevated RSV specific IgG1 antibody production. Th2 cytokine protein production by T cells showed no apparent change. Although IFN-γ production diminished in eosinophilic mice, it remained the major cytokine found in lung T cells. It was concluded that lung eosinophila can develop despite abundant IFN-γ production by local T cells, but is associated with a shift in the balance between Th2 and Th1 cytokine production.

Introduction

RSV is a ubiquitous paramyxovirus which causes relatively mild common cold symptoms in adults, but severe lower respiratory tract disease in young children (Heilman, 1990). At present, there are no effective, safe vaccines against RSV infection. Vaccination with formalin inactivated virus (FI-RSV) results in more severe (in some cases fatal) disease when natural RSV infection is encountered (Kapikian et al., 1969; Chin et al., 1969; Kim et al., 1969).

Subsequent studies in animal models show that immunizations with recombinant vaccinia viruses (rVV) expressing either G, F or M2 proteins reduce virus replication in the lower respiratory tract after infection with RSV. Immunization with rVV-G or rVV-F induces neutralizing antibodies which are sufficient to protect against virus replication, while vaccination with rVV-M2 induces protective T cell responses (Connors et al., 1992). F and M2 proteins both induce strong CTL responses, while G induces mainly CD4+ T cells and lung eosinophilia (Openshaw et al., 1992). Adoptive cell transfer of purified CD4+ G specific T cells into RSV infected mice results in similar eosinophilia and is associated with high mortality. These studies demonstrate that while CD4+ and CD8+ T cells clear RSV infection, they are also associated with immunopathology (Cannon et al., 1988; Graham et al., 1991; Alwan et al., 1992, 1994).

Since lung and blood eosinophilia is also seen in human FI-RSV vaccinees with augmented disease (Kim et al., 1969; Chin et al., 1969), it is possible that lung pathology induced by FI-RSV and G results from priming of Th2 cells during immunization. Th2 cells characteristically produce IL-4, IL-5, IL-10 and IL-13, and are important in inducing eosinophil and mast cell differentiation and proliferation. Th2 cells also promote humoral responses (especially IgG1 and IgE), are important in immunity to some parasitic infections, and contribute to allergic disease (Stevens et al., 1988; Mosmann & Coffman, 1989). Th1 cells make IL-2 and IFN-γ, and are mainly involved in delayed type hypersensitivity responses and the induction of IgG2a.

This functional dichotomy of helper T cells appears important in explaining the pathology of RSV infection.
RT–PCR analysis of lung tissue from RSV challenged mice immunized with Fl-RSV shows increased IL-4 mRNA expression and severe lung pathology which is diminished when mice are treated with antibodies to neutralize IL-4 and IL-10 (Connors et al., 1994). In addition, the pathology is reduced by co-administration of IL-12 during immunization to promote the development of Th1 responses (Tang & Graham, 1995). In vitro studies of protein specific responses show that G specific mouse splenocytes stimulated with RSV produce IL-4 and IL-5 (Alwan & Openshaw, 1993; Srikiatkhachorn & Braciale, 1997a), while similar stimulation of F and M2 specific cells results in Th1 cytokine production (Alwan et al., 1993). Peripheral blood T cells of healthy human donors also produce higher levels of Th2 cytokines following in vitro culture with G protein than with F protein (Jackson & Scott, 1996).

To clarify the mechanisms involved in vaccine induced immunopathology, we examined the kinetics of Th1 and Th2 cytokines following RSV challenge of mice sensitized to F, M2 and G using semi-quantitative RT–PCR and flow cytometric analysis of intracellular cytokines.

Methods

**Viruses and mice.** The human A2 strain of RSV and rVV (Stott et al., 1986; Wertz et al., 1987; Openshaw et al., 1990) were grown in Hep-2 cells, snap-frozen in liquid nitrogen and assayed for infectivity. All stocks were free from mycoplasma infection (tested by DNA hybridization, Gen-Probe Inc.). Groups of 10–12-week-old female BALB/c mice (Harlan Olac) were scarified with 3 × 10⁶ p.f.u. vaccinia virus expressing G, F or M2 proteins from RSV, or β-galactosidase (β-Gal) as a control. The mice were kept under pathogen free conditions in filter top cages for 3 weeks before being anaesthetized with ether and infected intranasally with 1–3 × 10⁶ p.f.u. RSV.

**Cell recovery.** Prior to RSV infection, and at various times after infection, bronchoalveolar lavage (BAL), the remaining lung tissue after BAL, mediastinal (MLN) and inguinal lymph nodes (ING) were recovered. Groups of four mice were injected intraperitoneally with pentobarbital (3 mg per mouse) and exanguinated via the femoral vessels. BAL was collected as described previously (Hussell et al., 1996). Samples were pooled from each group in cold RPMI medium containing 10% foetal calf serum, 2 mM/l-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (R10F). Single cell suspensions were prepared from lung tissue and MLN or ING lymph nodes (local to the sites of RSV infection and scarification, respectively). Mononuclear cells from lung tissue were isolated by centrifugation over Ficoll–Paque before cells from all sites were washed and resuspended at 1 × 10⁶/ml. Without in vitro stimulation, aliquots of 2 × 10⁶ cells were pelleted and snap-frozen for RNA extraction. Cytospins of 2 × 10⁶ BAL cells from individual mice were prepared, fixed and stained with Giemsa’s reagent for cytological analysis. The remaining cells were stimulated and stained for intracellular cytokines.

**Semi-quantitative RT–PCR of cytokine mRNA.** Total cellular RNA was isolated using RNEasy columns (QIAGEN) and RNA from the equivalent of 2 × 10⁵ cells used in cDNA synthesis reactions. A standard curve of fourfold dilutions of EL4 RNA, ranging from 2000 ng to 0.5 ng, as well as negative controls without RNA and without MMLV-RT (Gibco) were included. RNA was first annealed to 1 μg oligo(dT)12–18 (Pharmacia) at 60 °C for 5 min in a 9600 thermal cycler (Perkin Elmer) in cDNA buffer (75 mM KCl, 3 mM MgCl₂, 50 mM HCl buffer pH 8.3).

Subsequently, 20 mM DTT, cDNA buffer, 2 mM dNTPs, 400 U MMLV-RT, 40 U RNAse inhibitor (Promega) and RNAse free H₂O were added to each tube and incubated at 37 °C for 40 min. The reaction was inactivated by heating to 70 °C for 10 min.

PCR was carried out for 30 (β-actin and IL-10) or 35 cycles (IFN-γ, IL-2, IL-4 and IL-5) using 1/20 of the total amount of cDNA plus 0.2 mM dNTPs, 2.5 U AmpliTaq DNA polymerase (Perkin Elmer), MgCl₂ and primers as described previously (Hussell et al., 1996). Dot blots of PCR product were hybridized to 32P-labelled cytokine specific oligonucleotide probes, autoradiographed and β-emissions counted using a Matrix-96 β-plate reader (Canberra-Packard). Counts were compared against a regression analysis of an EL4 standard curve for each cytokine and allocated units of RNA content (i.e. EL4 RNA equivalents). The samples were normalized for β-actin levels and the corresponding cytokine data corrected.

**Lung virus tittes.** Homogenates of lungs from four individual infected mice were harvested 4 days after infection with RSV and assayed for infectivity as described (Stott et al., 1987).

**Serum immunoglobulin isotype ELISA.** ELISA plates were coated overnight at 37 °C with HEP-2 control and RSV antigens and then blocked with 1% rabbit serum for 2 h at 37 °C. The wells were washed three times with PBS–0.05% Tween 20 and twofold serial dilutions of sera (from 1:100 to 1:800) added. After 1 h at room temperature, the plates were washed and incubated with biotinylated anti-mouse IgG1 or IgG2a for 1 h at room temperature. The plates were washed and HRP conjugated avidin added for 40 min. Bound HRP was detected using o-phenylenediamine (OPD) substrate and the reaction stopped by addition of 2 M H₂SO₄. Absorbances were measured at 490 nm and readings from triplicate wells averaged. Background readings from HEP-2 antigen coated wells were subtracted. Relative shifts in the IgG1:IgG2a ratio were calculated for each mouse by dividing the absorbance reading for IgG1 by that obtained for IgG2a. Mean ratios were compared for each group and statistical analysis between groups carried out by analysis of variance.

**Intracellular cytokine staining and flow cytometry.** Freshly isolated cells were stimulated for 4 h at 37 °C with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 500 ng/ml ionomycin (Sigma). For the last 2 h of incubation 10 ng/ml brefeldin A (Sigma) was included. Cells were fixed in 2% formaldehyde (in hypertonic PBS) for 20 min at room temperature and then washed in PBS–1% BSA–0.1% sodium azide (PBS/BSA/azide). Cells were permeabilized with 0.5% saponin and then incubated with 5 μg per 10⁶ cells of phosphatidylethanolamine (PE) conjugated anti-IL-4 (11B11-PE, A. O’Garra, DNAX, USA) or anti-IL-5 (TRFK5-PE, Pharmingen) and FITC conjugated anti-IFN-γ (AN18-FITC, gift from A. O’Garra, DNAX, USA). Indirect staining of IL-10 was carried out as described previously (Hussell et al., 1996). Saponin was removed by washing with PBS/BSA/azide before addition of either quantum red conjugated rat anti-mouse CD4 (IgG2a, Sigma) or rat anti-mouse CD8 (IgG2a, Sigma). Lymphocytes were identified by their forward and side scatter properties on a Coulter EPICS Elite flow cytometer. Identical samples were stained with isotype matched control antibodies at each time point and used to define background gates for flow cytometry analysis.

Results

Enhanced immune responses to virus challenge of immunized mice

To determine the kinetics of immune responses to virus challenge, viable cell counts were obtained from lung and
Table 1. Cellular responses in BAL and MLN of RSV challenged mice

BAL and MLN cells recovered on day 4 and day 8 after RSV challenge of primed mice. Groups of four mice were immunized with 3 × 10⁶ p.f.u. rVV and 2 weeks later challenged with 3 × 10⁶ p.f.u. RSV. Viable cells were counted in pooled samples using light microscopy and trypan blue exclusion. The percentage of lymphocytes was determined by flow cytometry using forward and side scatter. BAL, bronchoalveolar lavage; MLN, mediastinal lymph node.

<table>
<thead>
<tr>
<th>Immunization (rVV)</th>
<th>Day after RSV challenge</th>
<th>Total viable cells recovered from BAL (× 10⁵ per mouse)</th>
<th>Percentage lymphocytes in BAL</th>
<th>Total viable cells recovered from MLN (× 10⁵ per mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>β-Gal</td>
<td></td>
<td>6.3</td>
<td>56.6</td>
<td>15.5</td>
</tr>
<tr>
<td>G</td>
<td>4</td>
<td>6.7</td>
<td>52.4</td>
<td>23.5</td>
</tr>
<tr>
<td>F</td>
<td>7.2</td>
<td>58.8</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>7.3</td>
<td>59.8</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>β-Gal</td>
<td>8</td>
<td>5.2</td>
<td>35.5</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>12.5</td>
<td>63.1</td>
<td>38.6</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>19.6</td>
<td>61.5</td>
<td>32.0</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>14.8</td>
<td>72.7</td>
<td>36.5</td>
<td></td>
</tr>
<tr>
<td>β-Gal</td>
<td>14</td>
<td>3.1</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>7.2</td>
<td>38.7</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>9.3</td>
<td>54.6</td>
<td>16.5</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>9.4</td>
<td>52.2</td>
<td>10.5</td>
<td></td>
</tr>
</tbody>
</table>

lymph node samples. In a representative experiment, approximately 5 × 10⁵ viable BAL cells per mouse (mainly macrophages) were recovered from uninfected control animals. Following virus challenge, the efflux of cells into lungs increased rapidly, peaking 8 days after infection. Lung infiltrates, and the percentage of lymphocytes present in BAL, were consistently greater in mice previously exposed to RSV proteins compared with those mice exposed to the control protein β-Gal (Table 1).

Differential cell counts of cytospins showed that while all BAL samples contained both small and large lymphocytes (as well as a small population of polymorphonuclear cells of generally < 5%), G was the only RSV protein which induced lung eosinophilia (approximately 14% of BAL cells) (data not shown).

MLN lymph nodes were not visible in uninfected animals, but rapidly increased in size following RSV infection (2.5, 7.0, 6.5 and 9.0 × 10⁵ cells per mouse recovered 24 h after RSV challenge from β-Gal, G, F and M2 primed mice respectively). The increase in MLN size preceded the increase in cellularity of BAL samples, suggesting that immune responses to viral antigen first occur in local nodes followed by trafficking of cells to the site of infection. The size of the nodes peaked 8 days after infection in all groups (Table 1).

The enhanced immune status of animals immunized with either G, F or M2 resulted in complete inhibition of infectious virus production measured in lung homogenates 4 days after challenge. The level of virus replication in β-Gal immunized mice (mean lung virus titre, 1.8 × 10⁴ p.f.u. at day 4) was consistent with titres previously obtained for mice of this age (Taylor et al., 1984; Stott et al., 1987).

Kinetics of cytokine mRNA induction

Fig. 1 shows dot blots of RT–PCR products from mRNA isolated from unseparated BAL cells (left panel) and the results are represented graphically (right panel) by comparison against an RNA standard isolated from EL4 T cells (Hussell et al., 1996). β-Actin mRNA levels were similar in all samples (data not shown). IFN-γ, IL-2, IL-4, IL-5 and IL-10 mRNAs were virtually undetectable in uninfected mice (day 0 samples). Following RSV infection, IFN-γ, IL-10, IL-4 and IL-5 mRNA transcription peaked in immunized mice at day 4. Prior sensitization of mice to RSV proteins was reflected in more rapid induction, and higher levels of cytokine mRNA transcription (compared with transcription in β-Gal primed mice).

IFN-γ mRNA increased dramatically following RSV infection and was easily detected at 24 h. The increase was more vigorous in F and M2 primed mice than in mice sensitized to G protein. Despite this strong induction of IFN-γ (a Th1 cytokine), IL-10 mRNA (classically a Th2 cytokine) was also strongly induced in F and M2 primed mice; however, the increase in IL-10 transcripts occurred at a slower rate than those of IFN-γ. Expression of another Th1 cytokine, IL-2, was markedly elevated within 24 h in M2 primed mice and by day 4 in F and G primed mice. IL-4 mRNA levels were low during primary infection (i.e. in β-Gal primed mice), but increased
transiently 4 days after infection of primed mice. Most notable were the elevated levels of IL-4 and IL-5 message in G primed mice. The increase in IL-5 transcription in G primed mice peaked 4 days after RSV infection, prior to maximal eosinophilia on day 7. Priming with G protein, therefore, increased the abundance of Th2 cytokines while reducing levels of Th1 cytokine mRNA transcription.

In MLN (day 4) and ING nodes (day 14), IFN-γ mRNA transcription was higher in M2 and F primed mice than in G and β-Gal primed mice (data not shown). The level of IFN-γ transcription was consistently higher in BAL samples than in nodes, but since the majority of replicating virus is thought to remain confined to respiratory epithelium, the induction of IFN-γ mRNA expression in both MLN and ING nodes suggests that RSV antigens are transported from the lung and presented to T cells at remote sites. Alternatively, activated T cells may traffic from the lung to other regions of the lymphoid system.

Basal levels of IL-4 transcription in the nodes of all groups of mice did not alter markedly following infection. Interestingly, IL-5 mRNA expression increased in the lung tissue (day 4, 76 pg) and ING nodes (day 8, 267 pg) of G immunized mice alone. IL-5 mRNA expression was not elevated, at any time during infection, in the lung tissue or nodes of F, M2 or β-Gal primed mice. IL-10 transcription in MLN peaked on day 4 after infection while expression in ING nodes increased similarly in all groups, peaking later at day 8 (data not shown). The induction of eosinophilia in G primed mice therefore correlates with elevated levels of IL-5 mRNA and reduced transcription of IFN-γ at multiple sites.

**Dominant Th or CTL responses associated with different immunizations**

The percentages of CD4$^+$ and CD8$^+$ cells in BAL or lymph node samples were determined by flow cytometry (Fig. 2). In F and M2 primed mice, CD8$^+$ T cells accumulated pro-
Fig. 3. Intracellular IFN-γ and IL-10 in T cells from various sites 7 days after RSV challenge of G immunized mice. Cells within the lymphocyte gate on forward/side scatter plots were analysed for CD4 or CD8 surface marker expression. Quadrant E gates were set on identical cells stained with isotype matched control antibodies. CD4+ (upper panel) and CD8+ (lower panel) cells were then analysed for intracellular IFN-γ and IL-10 expression. BAL, bronchoalveolar lavage cells; lung, Ficoll separated mononuclear cells from single cell preparations of whole lung; MLN, mediastinal lymph node cells; and ING, inguinal node cells.

Fig. 4. Intracellular IFN-γ and surface marker expression of BAL lymphocytes 14 days after RSV challenge of immunized mice. BAL cells were stained with quantum red conjugated anti-CD4 or anti-CD8 (Sigma) and FITC conjugated anti-IFN-γ (AN18). Quadrant gates were set to include 99% of identical cells stained with isotype matched control antibodies. Quadrant statistics are displayed in the top right hand corner of each dot plot.
gressively in BAL samples throughout the time course. In contrast, preferential accumulation of CD4+ T cells occurred in the lungs of G primed mice, reaching 56% of lymphocytes on day 14 after infection. The accumulation of CD8+ T cells in F and M2 primed mice, and CD4+ T cells in G immunized mice, occurred only in the lung since unchanged percentages of CD4+, CD8+ and B cells were detected in MLN or ING lymph nodes (data not shown). Approximately 10% or less of BAL cells stained for the B cell marker B220, leaving a substantial proportion of lymphocytes, particularly at early time points, which could not be classified as T or B cells. These are probably NK cells (T. Hussell & P. J. M. Openshaw, unpublished observations).

Flow cytometric analysis of T cell subset cytokine profiles

T cells from RSV infected immunized mice were analysed for their cytokine production patterns using intracellular cytokine staining and flow cytometry. In agreement with mRNA analysis, IFN-γ+ cells were more abundant at sites closest to the area of virus inoculation and replication (i.e. the response gradient was BAL > lung samples > MLN > ING). While a large proportion of both CD4+ (75.5%) and CD8+ (77.3%) BAL cells from G immunized mice were capable of IFN-γ expression, IFN-γ+ cells from MLN and ING lymph nodes were mainly CD8+ T cells. A significant proportion of the IFN-γ+ cells co-expressed IL-10 (Fig. 3).

T cell cytokine staining patterns of T cell subsets were compared during immunological responses induced by RSV challenge of mice immunized with β-Gal, G, F or M2. Despite clear differences in cytokine mRNA transcription in BAL samples, there was little difference in the percentage of IFN-γ+ cells in either CD4 or CD8 populations in different groups at early time points (data not shown). The high proportion of IFN-γ+ cells continued throughout the virus infection, even after the virus was cleared from the lung (day 4). Although similar percentages of IFN-γ+ T cells were present in the different groups of mice, the clear preference in recruitment of CD4+ T cells to the lung resulted in G immunized mice having more IFN-γ+/CD4+ and far fewer IFN-γ+/CD8+ T cells than either F or M2 primed mice. Differences between groups were more apparent later during infection. By day 14, G immunized mice had remarkably fewer IFN-γ producing CD8+ T cells than other mice (Fig. 4).

The percentages of IL-4 and IL-5 positive T cells detected by flow cytometry were low ( < 5% above background), and similar in all groups. Therefore neither the percentage of Th2 cytokine producing cells nor the total number of Th2 lymphocytes increased in animals with lung eosinophilia. In both T cell subsets, much lower percentages of IFN-γ+ cells were detected in MLN than in BAL. CD4+/IFN-γ+ MLN cells remained at around 8% throughout the infection, while CD4+ cells expressing Th2 cytokines comprised less than 1:5% of cells in all groups. Percentages of CD8+/IFN-γ+ MLN cells were similar in all groups (approximately 40%) and no apparent differences were detected in the proportions of CD8+ cells expressing Th2 cytokines (data not shown).

Ratios of RSV specific IgG1: IgG2a are increased following G immunization

To evaluate further the potential effects of Th1 and Th2 cytokine induction during RSV infection, ratios of RSV specific immunoglobulin isotypes were determined by ELISA. Antibody levels at days 0, 1 and 4 were below the sensitivity of the assay. In sera collected 14 days after RSV challenge, there was a significant increase (P = 0.05) in the ratio of IgG1:IgG2a isotypes in G primed mice compared with β-Gal, F or M2 primed mice (Fig. 5). This increase was apparent at all dilutions of sera.

Discussion

A greater understanding of the cell types and cytokines involved in immunological responses to RSV is required to identify protective, non-pathogenic components of immunity. Since intracellular cytokine staining has proved to be highly informative in studies of antigen specific T cell clones (Openshaw et al., 1995), we anticipated that this method would allow us to define Th1 and Th2 T cell subsets during RSV induced disease. In brief, we showed that G primed mice showed preferential accumulation of CD4+ T cells in the lungs, while immunization with F or M2 caused abundant CD8+ T cells to accumulate during RSV challenge. The preferential expansion of different T cell subsets only occurred at sites of

![Fig. 5. Serum immunoglobulin isotype ratios. Serum was collected from four individual mice at each time point following RSV challenge of immunized mice. Twofold serial dilutions of sera (from 1:100 to 1:800) were tested for RSV specific IgG1 or IgG2a antibodies using ELISA plates coated with HEp-2 control and RSV antigens. After addition of OPD substrate, absorbances were measured at 490 nm. Readings from three identical wells were averaged and the HEp-2 background subtracted from the RSV readings. The ratios between isotypes were then calculated for each mouse. Mean ratios were determined for each group and statistical analysis performed using analysis of variance. The results presented are from day 14 serum samples at 1:800 dilution. Error bars represent the standard error of the mean. Similar statistically significant results were obtained at other serum dilutions.](image-url)
virus replication. A remarkable finding is that IFN-γ is always more abundantly expressed than Th2 cytokines and that the different types of pathology after vaccination with single RSV proteins is associated with a relatively small shift in the balance of Th1/2 cytokines. Striking eosinophilic pathology is not matched by abundant local Th2 cell recruitment, but only by a small and delayed reduction in the accumulation of local IFN-γ producing cells.

When Th1 cytokine profiles were examined by RT–PCR, the results largely agreed with those obtained by intracellular cytokine staining, with a few important differences. Abundant IFN-γ mRNA was present in situations dominated by eosinophilia (a similar result to that seen in lung eosinophilia in mice vaccinated with FI-RSV; Waris et al., 1996). Abundant IFN-γ was also found in in vitro lung and spleen cells stimulated with RSV from G primed mice (Srikiatkhanchorn & Braciale, 1997a). In contrast to Srikiatkhanchorn’s study, however, we did observe less IFN-γ in G primed mice compared to those vaccinated with F or M2. Our different findings might be explained by the methods used since in our study we used short term or (in the case of RT–PCR analysis) no in vitro restimulation of cells. Rapid expression of IFN-γ during RSV challenge occurred in all mice at a time when relatively few lymphocytes had accumulated locally. Subsequent studies have shown that this early IFN-γ production is from NK cells (T. Hussell, unpublished observations).

The reduced levels of IFN-γ in G primed mice is interesting. An important difference between the different antigens used to vaccinate mice is that G neither induces nor is recognized by CD8+ T cells, whereas CD8+ T cells are strongly induced by vaccination with F and M2 (Openshaw et al., 1990, Srikiatkhanchorn & Braciale, 1997b). We have previously shown that CD8+ T cells are an important source of IFN-γ, which may explain the reduced levels observed by RT–PCR in G primed mice. Another possible explanation may be that Th2 cytokines downregulate IFN-γ in G vaccinated mice. Administration of IL-12 during vaccination with rVV-G reduces the level of eosinophilia following RSV challenge, while increasing total cell recruitment to the lung (Hussell et al., 1997). This enhanced cell recruitment in the presence of Th1 inducing conditions is consistent with that observed in F and M2 primed mice in our study.

Elevated levels of IFN-γ mRNA were not only detected at sites of RSV replication (e.g. the BAL and lung), but also at more distant sites. This may indicate responses to circulating viral antigens or homing of antigen-activated cells back to the original site of priming (i.e. the skin and local nodes in rVV vaccinated mice). The lower levels of IFN-γ and IL-10 detected at more distant sites may be due to variation in the level of antigen or transcription induced by circulating cytokines (Cockfield et al., 1993; Halloran et al., 1992). Similar graded responses have been described in Sendai and influenza virus infections, where replication is also largely confined to the respiratory tract (Doherty, 1995 and references therein). We consistently observed a direct correlation between the increases in transcription of IFN-γ and IL-10 mRNA. In addition, intracellular cytokine staining revealed that IL-10 is usually co-expressed with IFN-γ in both CD4+ and CD8+ T cells despite their classification as Th2 and Th1 cytokines, respectively. This co-expression has previously been shown in primary RSV infection (Hussell et al., 1996). Our present data suggest that IL-10 may function as a regulatory cytokine controlling IFN-γ expression during secondary as well as primary responses to RSV infection.

In this study, no IL-4 or IL-5 was detected in eosinophilic mice by intracellular cytokine staining but we did observe increases in both of these cytokines by RT–PCR compared to non-eosinophilic mice. This result raises an important issue regarding the technique used to define Th1/Th2 sub-populations in ex vivo samples. Unless the cellular response is phenotypically well characterized, the detection of IL-4 or IL-5 by RT–PCR does not necessarily mean that the RNA originates from Th2 cells. These cytokines are also produced by mast cells, basophils and eosinophils (Gordon et al., 1990). Cytokine production from such cells would contribute to the RNA signal but would not be seen on flow cytometric analysis. Other possible reasons for the discrepancy between the two techniques are differences in sensitivity and in the ability to quantify end product.

From these studies, it is clear that in vivo cytokine expression during RSV infection is more complex than studies based on in vitro culture would predict. The eosinophilia induced by vaccination with G protein does not occur as a result of priming of a numerically dominant population of CD4+ Th2 cells. Rather, these studies support the suggestion that overt differences in the composition of inflammatory leukocytes present at the site of virus infection arise through a shift in the balance between Th1 and Th2 cytokines. More specifically, very modest increases in Th2 cytokines result in lung eosinophilia even with continued abundant IFN-γ production from local T cells.

We thank A. O’Garra, DNAX, California, USA, for cytokine reagents; V. Manio and K. Davis, Becton Dickinson, San Jose, California, USA, for the PE conjugated AN18; G. Wertz, University of Alabama, Birmingham, USA, and co-workers for recombinant vaccinia viruses, and The Wellcome Trust, UK, for funding this work.

References


