Helper T Cell Recognition of Respiratory Syncytial Virus in Mice

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SUMMARY

In this study we aimed to define the protein and viral subtype specificities of helper T cells to respiratory syncytial virus (RSV). BALB/c mice were primed by infection with RSV, or with vaccinia viruses (VV) containing genes encoding several individual RSV proteins. Priming for T cell memory was assayed by stimulating spleen cells in vitro with different RSV isolates and measuring RSV-specific interleukin 2 (IL-2) release by T cells into supernatants using an IL-2-dependent CTLL cell line. Splenocytes from mice primed intranasally with RSV exhibited RSV-specific T cell memory, whereas those from unprimed mice did not. T cell recognition was in part specific to the strain of RSV used in priming and in part cross-reactive between RSV strains. Intraperitoneal priming with RSV fusion protein-expressing VV or nucleoprotein-expressing VV induced a stronger RSV-specific T cell response than the attachment glycoprotein-expressing VV which produced only slight T cell recognition. No T cell recognition of two non-structural proteins (1A and 1B) could be demonstrated.

INTRODUCTION

Respiratory syncytial virus (RSV) is the main cause of infantile bronchiolitis (Kim et al., 1973), which is the single commonest cause of hospitalization of young children in developed countries. In adults, RSV causes mainly upper respiratory symptoms, but also bronchitis and wheezing in some cases (Gardner, 1973). Those who recover from bronchiolitis are prone to recurrent wheezing in later life (Webb et al., 1985; McIntosh, 1976). The World Health Organization has selected RSV as a high priority for vaccine development.

Although high levels of RSV-specific antibody can protect both rodents (Prince et al., 1985; Taylor et al., 1984) and humans (Parrott et al., 1973) against infection, humoral immunity seems inefficient in preventing upper respiratory infection. Reinfection is common, occurring in 40 to 50% of adults in close contact with patients infected with RSV, despite neutralizing and complement-fixing serum antibody following previous infections. Formalin-inactivated (Kim et al., 1969; Fulginiti et al., 1969) and live attenuated (Wright et al., 1982) vaccines conferred no protection from natural infection, although vaccinated children developed neutralizing serum antibody. The highest morbidity and mortality is seen in infants 4 to 8 weeks old, although maternal antibody is present for at least 3 months (Chanock et al., 1970; Ward et al., 1983). Different isolates of RSV have been separated into two (Mufson et al., 1985) or three (Anderson et al., 1985) subgroups on the basis of reactivity with monoclonal antibodies, but antigenic variation does not seem to account for the recurrence of infection. Although many of the currently defined serological differences are due to variation in the major surface glycoprotein (G), variation of the fusion protein (F) and other proteins have been observed. The extent of variation will become clearer when more gene sequence data become available.

There are indications that T cell responses are important in RSV infection. Immunodeficient children are at high risk of complicated, prolonged and fatal RSV infection (Hall et al., 1986).
probably because of defective cellular immunity (Fishaut et al., 1980). Children recovering from bronchiolitis have been reported to have reduced proportions of OKT8-positive cells in the peripheral blood (the T killer subset), when compared to patients recovering from other forms of RSV infection (Welliver et al., 1984). Some cotton rats are immune to infection despite undetectable serum antibody (Prince et al., 1983), and transfer of primed T cells to persistently infected immunodeficient mice can lead to rapid viral clearance in the absence of antibodies (Cannon et al., 1987). Infants and children given formalin-inactivated RSV intramuscularly were unprotected from natural infection but showed lymphoproliferative responses in vitro to RSV antigens which were stronger than those in individuals naturally infected with RSV (Kim et al., 1980). Moreover, vaccinees were more severely affected by lower respiratory disease during subsequent natural infection with RSV than a control group given parainfluenza virus type 3 vaccine (Fulginiti et al., 1969; Kim et al., 1969). It is therefore important to study T cell responses to RSV and to find out which responses are beneficial and which might contribute to severe immunopathology.

RSV-specific major histocompatibility complex (MHC) class I-restricted cytotoxic T cells (T_c) have been reported in both mouse (Bangham et al., 1985) and man (Bangham & McMichael, 1986; Bangham et al., 1986) but helper T_h cell function has not been studied. We now report the induction of RSV-specific T_h cell memory by priming mice with live infectious RSV or with recombinant vaccinia viruses (VV) containing genes for individual RSV proteins. The generation of T_h cell memory in the spleen was measured by RSV-specific interleukin 2 (IL-2) release from T_h cells, using an IL-2-dependent CTLL cell line. Our results show that T_h cells are in part specific to the strain of RSV used to prime the mice and in part RSV cross-reactive between strains. Priming intraperitoneally with recombinant VV containing genes for the fusion (F VV) or nucleoprotein (N VV) of the A2 strain of RSV induced RSV-specific T_h memory cells, whereas the major surface attachment glycoprotein recombinant (G VV) and two recombinants encoding non-structural proteins (1A VV and 1B VV) induced little or no detectable recognition.

**METHODS**

**Viruses.** The A2 and 8/60 strains of RSV were provided by Dr E. J. Stott (Institute for Research on Animal Diseases, Compton, U.K.) and the RSN-2 and RSS-2 strains by Professor C. R. Pringle (University of Warwick, Coventry, U.K.). Stock viruses were grown in HEp-2 cells as previously described (Bangham et al., 1985). Control HEp-2 antigen was produced by mock infection. Virus infectivity was assayed in HEp-2 cells grown in 96-well flat-bottomed microtitre plates infected overnight and stained for viral antigens by an immunoperoxidase method (Cannon, 1987). A monoclonal antibody to fusion protein (13-1, kindly provided by Dr B. Fernie, Georgetown University Schools of Medicine and Dentistry, Rockville, Md, U.S.A.) was used and detected using rabbit anti-mouse IgG antibody conjugated to peroxidase to form an insoluble product of 3-amino-9-ethylcarbazole. The RSV isolates used were all free of contamination with *Mycoplasma* spp.

The construction of the recombinant vaccinia viruses has previously been described (Ball et al., 1986; Wertz et al., 1987). In brief, a cDNA copy of the coding sequence for a specific protein from A2 strain RSV was cloned behind the WR strain VV 7-5K promoter and recombined according to the protocol described by Ball et al. (1986). The nucleoprotein recombinants VN 125 and VN 333 were respectively low and high expressors of RSV nucleoprotein. The F protein recombinants VF 325 and VF 317 both expressed RSV F protein, but differed in sequences preceding the start of the F gene. V1A 4 and V1B 11 were VVs respectively encoding the 1A and 1B non-structural proteins. All genes were in positive orientation with respect to the VV promoter. Stocks were grown in and assayed on HEp-2 cells as previously described (Bangham et al., 1986). Virus was released by sonication of the washed cells and stored at −70 °C in 0·5% bovine serum albumin in Dulbecco's phosphate-buffered saline 'A' giving material containing 2 × 10^8 to 5 × 10^8 p.f.u./ml of live virus.

**Infection of mice.** BALB/c mice were bred under specific pathogen-free conditions at the National Institute for Medical Research (London, U.K.). Intranasal infection was performed by instillation of 50 μl of medium containing 2 × 10^5 p.f.u. of RSV or 5 × 10^6 p.f.u. of VV recombinant. Intraperitoneal infection with VV recombinants was by injection of 2 × 10^7 p.f.u. in 100 μl. Cutaneous infection was performed by scarifying a shaved area over the base of the tail and rump with 2·5 × 10^6 to 3·5 × 10^6 p.f.u. of VV in 10 μl. Fifty to 100 gentle scratches under light anaesthesia produced skin lesions appearing at 3 days.

**Release of IL-2 by T_h cells.** Spleens were removed 4 to 8 weeks after priming and disrupted by pressing through steel gauze. These responder cells were washed and resuspended in RPMI 1640 with penicillin (60 μg/ml),
**RSV-specific T_h cell memory**

Table 1. *Inhibition of IL-2 release by antibody to CD4 (L3T4)*

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody and concentration</th>
<th>³H-TdR incorporation (c.p.m. ± s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>820 ± 22</td>
</tr>
<tr>
<td>HEp-2</td>
<td>None</td>
<td>780 ± 51</td>
</tr>
<tr>
<td>RSV</td>
<td>None</td>
<td>3780 ± 32</td>
</tr>
<tr>
<td>RSV</td>
<td>Anti-L3T4 0.5 µg/ml</td>
<td>820 ± 30</td>
</tr>
<tr>
<td>RSV</td>
<td>Anti-L3T4 5 µg/ml</td>
<td>620 ± 28</td>
</tr>
<tr>
<td>RSV</td>
<td>Anti-Lyt2 ascites 1:5000</td>
<td>3520 ± 121</td>
</tr>
<tr>
<td>RSV</td>
<td>Anti-Lyt2 ascites 1:500</td>
<td>3960 ± 60</td>
</tr>
</tbody>
</table>

* Splenocytes (1-5 × 10⁵/0.2 ml) from mice primed by intranasal infection with RSV A2 were stimulated with viral or control antigen with or without monoclonal antibodies to Lyt2 (HO 2-2; Gottlieb et al., 1980) or L3T4 [YTS 191-1 (Cobbold et al., 1984), kindly provided as an ammonium sulphate precipitate by Drs S. Cobbold and H. Waldmann] T cell surface antigens. The antibody concentrations chosen are at, or in excess of, those shown to inhibit fully growth and proliferation of influenza-specific T_c or T_h cells (G. Taylor & B. A. Askonas, unpublished). The supernatants were taken at 72 h and IL-2 content tested by CTLL stimulation. Background CTLL ³H-TdR incorporation (147 c.p.m.) has been subtracted. CTLL uptake induced by 10% crude IL-2 was 24500 c.p.m. in this assay.

IL-2 assay. IL-2-dependent CTLL cells (Gillis & Smith, 1977) were maintained in RPMI with 10% FCS, antibiotics, glutamine and IL-2 in the form of 10% culture supernatant from rat splenocytes stimulated with concanavalin A (crude IL-2). The assay was performed by washing the CTLL cells three times and suspending at 10⁵ cells/ml in serum-free RPMI. Fifty μl (5 × 10⁵ cells) was incubated in 96-well round-bottomed microtitre plates with 50 μl of undiluted or diluted test sample for 20 h before addition of 0.5 μCi of [³H]thymidine (³H-TdR). After 4 to 6 h the cellular DNA was harvested onto filters, dried and assayed by liquid scintillation counting (Beckman LS-7500). Results were calculated as the mean c.p.m. of three identical wells after subtraction of counts obtained when CTLL cells were incubated with medium alone (typically 200 to 400 c.p.m.). Individual values were usually within 5% of the mean. In each assay, wells containing 10% crude IL-2 acted as positive controls, typically inducing 25000 c.p.m. of ³H-TdR incorporation.

Levels of IL-2 in microwell spleen cell culture supernatants were never high, inducing up to 20% of maximum ³H-TdR incorporation. Dilution of supernatants always reduced CTLL stimulation in direct proportion to the dilution factor. Since prozone effects were never seen, we report only the results of assays on undiluted supernatants. Using this method, antigen-specific increases in ³H-TdR incorporation provides an index of RSV-specific IL-2 release by T_h cells.

**RESULTS**

**RSV stimulation of IL-2 release**

Splenocytes from mice primed by intranasal infection with RSV and stimulated in vitro with RSV secreted IL-2, whereas normal splenocytes or those from mice primed intranasally with control HEp-2 cell material did not. IL-2 release was inhibited by the presence of monoclonal antibody to the L3T4 cell surface antigen in the splenocyte cultures, whereas monoclonal antibody to the Lyt2 antigen had little or no effect (see Table 1). IL-2 production was therefore attributable to L3T4⁺ (T_h) cells. Gamma irradiation (800 rad) of the responder cells abolished the response. The presence of live RSV did not interfere with concanavalin A-induced or spontaneous IL-2 release from primed or normal splenocytes (results not shown).
Fig. 1. RSV-specific T<sub>H</sub> cell recognition. Mice were primed intranasally with A2 or 8/60 RSV. Spleen cells were stimulated in vitro 3 (a) or 7 (b) weeks later with 8/60 (hatched bars), A2 (solid bars) or control uninfected cells (open bars). [3H]Thymidine incorporation into CTLL cells reflects IL-2 release by helper T cells. Error bars show the S.E.M. of the CTLL assay performed in triplicate on pooled supernatants.

Fig. 2. Time course of RSV-induced IL-2 production by primed T<sub>H</sub> cells. Mice were primed with A2 virus intranasally. Three weeks later splenocytes were stimulated in vitro with A2 (○), RSS-2 (△) and RSN-2 (○) virus. Aliquots of supernatant were taken on days 1 to 5 inclusive. Ordinate gives days of incubation in vitro and abscissa the [3H]-T<sub>dR</sub> incorporation into CTLL cells in the presence of the different samples, reflecting IL-2 content. The S.E.M. of the CTLL assay is shown.

Specificity of T<sub>H</sub> cells for different RSV strains

In order to show whether the RSV-specific T<sub>H</sub> cells are cross-reactive between strains of RSV, mice were primed with either A2 (group 3 of Anderson et al., 1985) or 8/60 (group 2). Splenocytes from these mice were cultured with cells infected with each of the priming strains. Fig. 1 shows that IL-2 release from splenocytes taken 3 (a) and 7 (b) weeks after intranasal infection was greater when stimulated with the virus used in priming, but there was considerable RSV strain cross-reactivity. A time course of IL-2 production is shown in Fig. 2. In this experiment, splenocytes from mice primed with A2 virus (group 3) were stimulated with A2, RSS-2 (unassigned by Anderson et al., 1985) and RSN-2 (group 2) virus. T<sub>H</sub> cells were again partly cross-reactive between RSV strains. The highest levels of IL-2 production (above background) were seen 2 or 3 days after stimulation. Optimal levels of RSV-specific IL-2 were generally found with 10<sup>6</sup> responder cells per ml at 2 days and 5 x 10<sup>5</sup>/ml at 3 days. We found the Long strain of RSV (group 1) to be unsuitable for stimulation in vitro since it caused aggregation of cells and abolished IL-2 release.

Induction of T<sub>H</sub> cell memory specific for different RSV proteins

In order to demonstrate the protein specificity of T<sub>H</sub> cells, mice were primed with the VV recombinants containing genes coding for single proteins of the human A2 strain of RSV. Priming with F VV or N VV successfully induced RSV-specific T<sub>H</sub> cell memory in the spleen, whereas G VV, IA VV and 1B VV were poor or ineffective in this respect. Fig. 3 shows the results of one representative assay on mice primed by dermal sacrifice. Although the
RSV-specific Th cell memory

Fig. 3. Th recognition of individual RSV proteins. RSV-induced IL-2 release from the splenocytes of mice primed by intranasal infection with A2 RSV or by scarification with G, F, N, 1A or 1B VVs. Open bars show CTLL 3H-TdR incorporation induced by supernatants of cultures in vitro with control HEp-2 antigen and solid bars that induced by A2 RSV culture. The S.E.M. of the CTLL assay is shown.

background levels of IL-2 were increased approximately twofold in cultures of splenocytes from mice primed intraperitoneally with VV, augmentation by RSV was highly reproducible. The mean stimulation index (ratio between antigen and antigen-independent IL-2 release) ± S.E.M. induced by the panel of viruses in a series of experiments is as follows: F VV, 3.1 ± 0.4, P < 0.0001 (paired t-test on eight experiments); N VV 1.9 ± 0.2, P < 0.0001 (n = 8); G VV 1.3 ± 0.3, P < 0.05 (n = 8). The 1A and 1B VVs induced no significant priming for RSV-specific Th cells. These stimulation indices compare with 5.1 ± 0.9, P < 0.0001 (n = 16) for splenocytes from RSV-primed mice. Intranasal infection with N VV or F VV did not induce detectable Th cell memory in the spleen (not illustrated). Results from mice primed with VVs expressing high or low levels of N protein or the two F VVs were indistinguishable.

DISCUSSION

Th cells play a central role in coordinating the immune response. They have fine antigenic specificity and provide stimuli for growth and functional maturation of T and B cells. In addition, they are directly involved in initiation of inflammatory responses. These functions depend both on cellular contacts and on secretion of soluble factors. The secretion of such factors (e.g. interleukins) allows Th cell function to be monitored in mixed cell cultures. In this report we have demonstrated RSV-specific IL-2 release by spleen cells from mice primed with RSV or VV vectors. This IL-2 release provides a convenient index of Th cell recognition and allows the specificity of Th cell function to be explored.

Firstly, we have examined the specificity of Th cells from mice primed by infection with different strains of human RSV. Although Th recognition was in part specific to the variant used in priming, we also demonstrated cross-reactive Th recognition. A previous study of strain recognition by Tc cells (Bangham & Askonas, 1986) showed cross-reactive recognition by polyclonal activated Tc cells. The specificity of T cell lines and clones is now being studied (M. J. Cannon, unpublished observations).

Secondly, we have used a panel of artificial recombinant viruses to explore the protein specificity of Th recognition. These VV recombinants contain genes for (and express) single RSV proteins. Infection of mice with these recombinants induces immune recognition of natural RSV which is specific to the chosen protein. We have recently shown (Pemberton et al., 1987) that the F VV is more efficient than the N VV in generating Tc memory cells specific to RSV,
whereas the G VV and other partial matrix protein gene or non-structural VVs are ineffective in this respect. Our present results show a similar ranking of RSV proteins with regard to Th cell recognition. This result was unexpected, since it implies associative recognition of the various proteins to be biased similarly by both MHC class I and class II surface antigens.

The protective immunity induced by VV recombinants encoding the RSV F and G proteins has been described both in mice (Wertz et al., 1987; Stott et al., 1986) and cotton rats (Olmsted et al., 1986; Elango et al., 1986). The studies in mice showed that infection with 10⁷ p.f.u. of F or G VV intraperitoneally protected mice from intranasal infection with natural RSV 3 weeks after priming. This conclusion was reached because RSV could not be recovered from the lungs 5 days after intranasal challenge. Further studies have now shown that this protection is associated with increased histological changes in the lungs (E. J. Stott, personal communication). Since the severity of RSV-induced bronchiolitis is reflected by the severity of histological abnormalities rather than the quantity of recoverable infectious virus, the term 'protection' requires qualified use. These studies showed that RSV replication could be inhibited by priming with F and G VV's, either intraperitoneally or intranasally. In this study we have shown Th cells in the spleen after intraperitoneal or cutaneous infection, but none after intranasal priming.

Serum antibodies to G protein have been demonstrated after natural RSV infection in man (Wagner et al., 1986) and following G VV infection of rabbits and mice (Stott et al., 1986). The G protein is thought to be unique among known viral surface proteins in being composed largely of oligosaccharide, much of which is O-linked (Wertz et al., 1985; Gruber & Levine, 1985). It has therefore been suggested that the G protein might act as a carbohydrate antigen and generate antibody independent of T cell help (Ward et al., 1983). However, recent analysis of the anti-G protein IgG subclasses in young children (Wagner et al., 1986) and mice (E. J. Stott, personal communication) suggests that G protein acts as a typical proteinaceous antigen and generates subclasses similar to those directed against F protein. Since this response depends on T cell help, our demonstration of weak G protein Th cell recognition needs explanation. Possible reasons include: (i) help may be provided by cells reacting to other viral proteins (Scherle & Gerhard, 1986); (ii) small numbers of Th cells may be sufficient for antibody production but remain undetected by our methods of assay; (iii) G protein-specific Th cells may be present in tissues other than the spleen; (iv) Hendricks et al. (1987) describe a soluble low Mr form of G protein and speculate that such a protein might saturate antigen receptors on virus-specific T cells. It seems improbable that such binding could interfere with T cell recognition of antigen in association with class II MHC gene products on the surface of antigen-presenting cells; (v) Th cell clones have been described which produce no IL-2, but secrete IL-3 and other factors (Mosmann et al., 1986). Such activity would not have been detected by our methods. The relative importance of these factors is open to speculation.

Many of the problems raised by clinical studies of RSV infection remain unsolved. For example, the frequency of reinfection, the dissociation between demonstrable humoral immunity and protection and the enhanced disease following vaccination are all poorly understood. Further analysis of T cell immunity to RSV may explain some of these features. To this end, we are now examining the beneficial and harmful effects of T cell lines and clones in RSV-infected mice. We hope that such studies will aid the development of successful vaccines against RSV in higher mammals.

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