Cytotoxic Lymphocytes in the Lungs of Mice Infected with Respiratory
Syncytial Virus

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SUMMARY
Mice infected with respiratory syncytial virus (RSV) developed cytotoxic lymphocytes in the lungs, which lysed RSV-infected, but not uninfected cells. Cytotoxic activity was greatest 7 to 9 days after infection, was virus-specific, MHC-restricted and abolished by treatment of lymphocytes with anti-Thy 1.2 or with anti-Lyt 2.2 sera and complement. There was a close temporal relationship between the appearance of these cytotoxic lymphocytes in the lung and clearance of virus. In contrast, RSV persisted in the lungs of athymic (nude) mice and such animals failed to develop RSV-specific cytotoxic lymphocytes. Thus, cytotoxic T-cells may have an important role in recovery from RSV infection.

INTRODUCTION
Respiratory syncytial virus (RSV) is a major cause of respiratory disease in man and cattle (Stott & Taylor, 1984). Epidemiological and experimental data indicate that antibody can protect against RSV infection (Lampechet et al., 1976; Glezen et al., 1981; Ogilvie et al., 1981; Ward et al., 1983; Prince et al., 1983; Walsh et al., 1984; Taylor et al., 1984a). However, there are inconsistencies. Thus, most severe disease frequently occurs within the first few months of life when infants possess moderate levels of maternal antibody (Parrott et al., 1973; Bruhn & Yeager, 1977). In such children, neither complement-fixing (CF) nor neutralizing serum antibody correlated with resistance to RSV. In addition, CF and neutralizing antibodies induced by a formalin-inactivated vaccine failed to protect against either infection or disease (Kapikian et al., 1969; Kim et al., 1969; Fulginiti et al., 1969). These discrepancies have stimulated investigations into the role of cell-mediated immunity in RSV infection using lymphocyte transformation and leukocyte migration inhibition tests (Scott et al., 1978; Bertotto et al., 1980). The importance of cytotoxic T-lymphocytes (CTL) in recovery from many viral infections, including those caused by other paramyxoviruses, has also encouraged investigations into their role in RSV infections. Leukocytes capable of lysing RSV-infected cells, but not uninfected cells, have been demonstrated in the lungs of RSV-infected cotton rats (Sun et al., 1983a). However, the cytotoxic activity was neither virus-specific nor restricted by the major histocompatibility complex (MHC), and it was proposed that the effector cells were either macrophages or natural killer (NK) cells. Nevertheless, there was a good correlation between the appearance of these cytotoxic leukocytes and clearance of RSV (Sun et al., 1983a, b). These studies were hampered by the fact that the cotton rats were not inbred, making studies of MHC restriction difficult. Since the mouse is susceptible to RSV infection (Prince et al., 1979; Taylor et al., 1984b), the present study employed BALB/c and C57BL/6 mice infected with human RSV to investigate the development of CTL in RSV infection.

METHODS
Mice. Specific pathogen-free (SPF) BALB/c, C57BL/6, nu/nu (athymic) BALB/c mice and nu/+ (normal) BALB/c litter-mates of the MRC Laboratory Animal Centre's category 4 standard were obtained from the SPF unit at the Institute for Research on Animal Diseases, Compton and from the National Institute for Medical Research, Mill Hill, London, U.K. Mice weighing 20 to 25 g were inoculated intranasally (i.n.) with...
approximately 10⁴ p.f.u. of the A2 strain of human RSV as described previously (Taylor et al., 1984b). Virus in tissue homogenates was assayed on secondary calf kidney cells as described elsewhere (Taylor et al., 1984b).

Preparation of effector cells. Lung lymphocytes were prepared by chopping lungs, from 10 to 15 mice, into small pieces and incubating them in 15 ml of 90 U/ml of collagenase (Sigma type C-9891 or type C-2139) in RPMI 1640 containing 25 mM-HEPES buffer, 100 U/ml penicillin and 100 μg/ml streptomycin (RPMI-HEPES) with 5% foetal calf serum (FCS) (Davies & Parrott, 1981b). After 90 min incubation at 37 °C with strong agitation, lung fragments were disrupted using a syringe. Following filtration through sterile muslin, lymphocytes were purified on a Percoll (Pharmacia) step gradient (Davies & Parrott, 1981a). After washing in phosphate-buffered saline (PBS), lung lymphocytes were finally resuspended in RPMI-HEPES medium containing 10% FCS. Approximately 2 × 10⁶ purified lymphocytes were produced from the lungs of each mouse.

Treatment of lymphocytes with antisera and complement. Lung lymphocytes were obtained from mice infected 7 days previously with RSV. Aliquots of 5 × 10⁷ lung lymphocytes were resuspended in 1 ml RPMI-HEPES medium containing 2% FCS and a 1:1600 dilution of Thy 1.2 antiserum (F7D5 monoclonal IgM antibody; Olac 1976 Ltd, Bicester, U.K.) or in medium containing a 1:640 dilution of Lyt 2.2 antiserum [HO 2.2 monoclonal IgM antibody, originally described by Gottlieb et al. (1980) and generously provided by Dr M. Cannon, N.I.M.R., Mill Hill, U.K.]. After incubation at 4 °C for 45 min, the cells were washed and resuspended at 1 × 10⁷ cells/ml in RPMI–HEPES medium containing 2% FCS and a 1:8 dilution of rabbit complement (C'). After incubation at 37 °C for 45 min, the cells were washed twice and resuspended to give 1 × 10⁷ viable cells per ml. As controls, lung lymphocytes were treated with either medium alone or with 1:8 dilution of rabbit complement. Lung lymphocytes treated in this way were used as effector cells in the cytotoxicity assay.

Target cells. A continuous line of BALB/c cells and the same line persistently infected with the Long strain of RSV, BCH-4 (Fernie et al., 1981) were obtained from Dr B. Fernie, Georgetown University, Rockville, Md., U.S.A. Both cell lines were maintained in medium containing equal parts of MEM with Earle’s salts and basal Eagle’s medium with Hanks’ salts, 10% heated FCS and 2 mM-glutamine as described previously (Fernie et al., 1981). The BALB/c cell line was infected with parainfluenza type 3 (Pi-3) virus at a multiplicity of 10 p.f.u./cell for 24 h.

Mouse embryo fibroblasts (MEF) were obtained from 12 to 15 day embryos of SPF BALB/c and C57BL/6 mice, and grown in Eagle’s MEM with 10% heated FCS, 2 mM-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. MEF were infected with the A2 strain of RSV at a multiplicity of 20 p.f.u./cell for 24 h.

YAC-1 cells were obtained from Flow Laboratories and grown in RPMI–HEPES with 10% FCS.

Chromium release assay. Suspensions of infected and control target cells were prepared by trypsinization of monolayers. Cells (10⁶) were labelled with approximately 200 μCi Na⁵¹CrO₄ (Amersham) for 1 h at 37 °C. Lysis of 10⁴ ⁵¹Cr-labelled target cells was assayed after 6 h by estimating the amount of ⁵¹Cr released by effector lymphocytes at various effector to target cell ratios. Background spontaneous release of ⁵¹Cr was 10% for BCH-4 cells, 6% for the uninfected BALB/c line, 18% for RSV-infected MEF cells and 8% and 16% for control BALB/c and C57BL/6 MEF cells respectively. The percentage specific release of ⁵¹Cr = [(c.p.m. experimental – c.p.m. spontaneous release)/c.p.m. total – c.p.m. spontaneous release] × 100. Each value was the mean of three replicates. The total releasable ⁵¹Cr was counted after lysis of target cells by 1% sodium lauryl sulphate. One lytic unit (L.U.) was taken as the number of effector cells necessary to cause 33% specific release (Cerottini & Brunner, 1974).

RESULTS

Appearance of cytotoxic effector cells in lungs

Lung lymphocytes from uninfected mice caused little ⁵¹Cr release from either BCH-4 or uninfected BALB/c line cells (<5% at effector to target cell ratios of 50:1). However, after infection of mice, significant cytotoxic activity against BCH-4 cells was observed at day 5 (Fig. 1). Peak cytotoxic activity was observed at day 9 and then declined, although significant cytotoxic activity was still present 14 days after infection (Fig. 1). Lysis of uninfected BALB/c cells remained at a low level throughout 14 days after infection, and NK cell activity, as determined by lysis of YAC-1 cells, showed only a slight increase from day 7 to day 14 (Fig. 1). The appearance of lung lymphocytes cytotoxic for RSV-infected cells coincided with the clearance of RSV from the lungs (Fig. 1).

Virus specificity of cytotoxic lung lymphocytes

Lymphocytes from the lungs of mice infected 7 days previously with RSV lysed RSV-infected cells (BCH-4) but not uninfected BALB/c cells nor BALB/c cells infected with Pi-3 virus (Table 1). Lung lymphocytes from uninfected mice caused little ⁵¹Cr release from BCH-4, uninfected BALB/c or Pi-3 virus-infected BALB/c cells.
Cytotoxic lymphocytes to RSV in mice

Fig. 1. Cytotoxic activity and virus infectivity in the lungs of BALB/c mice at various times after intranasal infection with RSV. ○, L.U. against BCH-4 target cells; □, L.U. against YAC-1 cells; △, virus infectivity (log_{10} p.f.u./g).

Fig. 2. Virus infectivity in the lungs of normal (●) and nude (○) BALB/c mice at various times after i.n. infection with RSV.

Table 1. Virus specificity of cytotoxic lymphocytes from lungs of RSV-infected BALB/c mice

<table>
<thead>
<tr>
<th>Target cells</th>
<th>% Specific release*</th>
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<tbody>
<tr>
<td></td>
<td>RSV-infected mice†</td>
</tr>
<tr>
<td>BCH-4</td>
<td>48.6 ± 2.6</td>
</tr>
<tr>
<td>Control BALB/c</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Pi-3 BALB/c line</td>
<td>-0.2 ± 0.6</td>
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* Percentage specific lysis expressed as means ± standard deviation of three replicates for 6 h assay, 50:1 effector to target cell ratio. Effector cells were prepared from groups of 10 mice.
† Mice inoculated 7 days previously with approx. 10^4 p.f.u. RSV i.n.
‡ Mice inoculated 7 days previously with approximately 10^4 p.f.u. RSV i.n. Effector to target cell ratio was 50:1.

Table 2. MHC restriction of cytotoxic lymphocytes from mouse lungs

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Antibody complement†</th>
<th>Mouse‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV BALB/c MEF</td>
<td>34.0 ± 3.1</td>
<td>RSV BALB/c</td>
</tr>
<tr>
<td>Control BALB/c MEF</td>
<td>3.3 ± 1.0</td>
<td>22.6 ± 1.5</td>
</tr>
<tr>
<td>RSV C57BL/6 MEF</td>
<td>19.9 ± 1.4</td>
<td>1.5 ± 1.1</td>
</tr>
<tr>
<td>Control C57BL/6 MEF</td>
<td>5.3 ± 0.7</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>BCH-4</td>
<td>39.6 ± 0.4</td>
<td>6.0 ± 0.8</td>
</tr>
<tr>
<td>BALB/c line</td>
<td>0.6 ± 0.4</td>
<td>40.7 ± 2.3</td>
</tr>
</tbody>
</table>

* Percentage specific lysis expressed as mean ± standard deviation of three replicates for 6 h assay.
† 1/10 dilution of gnotobiotic calf anti-RSV serum and 1/8 dilution of rabbit C'.
‡ Mice inoculated 7 days previously with approximately 10^4 p.f.u. RSV i.n.
§ ND, Not done.
Fig. 3. Cytotoxic activity of lung lymphocytes obtained from mice infected 7 days previously with RSV after treatment with rabbit C' alone (●, ○), Thy 1.2 antiserum and C' (▲, △) or Lyt 2.2 antiserum and C' (■, □), against (a) RSV-infected BCH-4 cells and (b) uninfected BALB/c cells.

Table 3. Cytotoxic lymphocytes in the lungs of normal and nude BALB/c mice*

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Normal mice</th>
<th>Nude mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCH-4</td>
<td>6.4</td>
<td>0.07</td>
</tr>
<tr>
<td>Control BALB/c line</td>
<td>0.37</td>
<td>0.54</td>
</tr>
<tr>
<td>YAC-1</td>
<td>5.0</td>
<td>12.9</td>
</tr>
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</table>

* Groups of six to 11 mice, inoculated 7 days previously with approximately 10^4 p.f.u. RSV i.n.

MHC restriction of cytotoxic lung lymphocytes

Lung lymphocytes from BALB/c mice infected 7 days previously caused lysis of RSV-infected BALB/c MEF cells and BCH-4 cells, but not of uninfected BALB/c cells (Table 2). In contrast there was no significant lysis of either RSV-infected C57BL/6 MEF cells or uninfected C57BL/6 MEF cells. Lung lymphocytes from RSV-infected C57BL/6 mice caused significant lysis only of RSV-infected C57BL/6 MEF cells. There was no significant lysis of any type of target cell by lung lymphocytes from uninfected mice. Lysis by antibody and complement indicated that the three types of RSV-infected target cells carried viral antigen on their surfaces; however, RSV-infected C57BL/6 MEF cells appeared to be poorer targets than either BALB/c MEF cells or BCH-4 cells.

Effect of Thy 1.2 and Lyt 2.2 antisera

Lung lymphocytes obtained from mice infected 7 days previously with RSV were treated with Thy 1.2 antiserum and C', Lyt 2.2 antiserum and C', C' alone, or left untreated prior to cytotoxicity assay. RSV-infected cells (BCH-4) were lysed by lung lymphocytes that were sensitive to treatment with Thy 1.2 antiserum and C' and with Lyt 2.2 antiserum and C' (Fig. 3). Lysis of BCH-4 cells by lymphocytes treated with C' alone was no different from lysis by untreated lung lymphocytes.
Normal BALB/c mice and nude BALB/c mice were inoculated i.n. with 10^4 p.f.u. of RSV. Groups of five mice were killed at intervals to determine the amount of virus present in the lungs. There was no significant difference in the amount of virus recovered 5 days after inoculation from normal and athymic mice (Fig. 2). However, by day 13, virus was undetectable in the lungs of normal mice. In contrast high titres of virus persisted in the lungs of athymic mice for at least 19 days (Fig. 2). Virus was not recovered from brain, liver, spleen or kidney of either normal or nude mice.

The lungs of normal and athymic mice were examined 7 days after RSV infection for the presence of lymphocytes cytotoxic for RSV-infected cells, and for NK cells (Table 3). There was little or no lysis of RSV-infected cells by lymphocytes from nude mice. In contrast, cytotoxic activity against RSV-infected BCH-4 cells was over 60-fold greater in normal mice than in nude mice 7 days after infection with RSV. There was no significant lysis of uninfected BALB/c cells. Although NK cell activity, as determined by lysis of YAC-1 cells, was present in both groups of mice in this experiment, there were 2.5-fold more lytic units in nude mice than in their normal litter-mates.

**DISCUSSION**

After i.n. inoculation of mice with RSV, lymphocytes capable of lysing RSV-infected, but not uninfected cells, were demonstrated in the lungs. The cytotoxic activity was greatest 7 to 9 days after infection and was virus-specific. Although RSV-infected allogeneic cells were poor targets, there was evidence that lysis of RSV-infected cells was MHC-restricted. In addition, cytotoxic activity was abolished by both Thy 1.2 antiserum or Lyt 2.2 antiserum and complement. These properties are characteristic of virus-specific CTL. This is the first demonstration of CTL in the lung active against RSV. Our findings contrast with the demonstration of cytotoxic cells in the lungs of RSV-infected cotton rats, which were neither virus-specific nor MHC-restricted in their activity (Sun et al., 1983a). The characteristics of the cytotoxic response in the lungs of cotton rats were similar to those of NK cells. There was a correlation between their presence and clearance of RSV from the lungs, suggesting a role for these cells in recovery from RSV infection (Sun et al., 1983a). In BALB/c mice, however, the increase in CTL in the lungs occurred in the absence of any marked increase in NK cell activity, as measured by lysis of YAC-1 cells, and coincided with clearance of RSV from the lungs, suggesting a role for CTL in recovery from RSV infection. This suggestion is also supported by the findings that nude mice failed to develop RSV-specific CTL, and virus persisted in their lungs for at least 3 weeks after inoculation despite high levels of NK cell activity. In fact, as has been shown previously (Luini et al., 1981) NK cell activity was greater in the lungs of nude mice than in normal mice (Table 3). However, since antibody titres were also reduced in nude mice (unpublished observations), the relative roles of CTL and antibody in the clearance of RSV is not yet clear.

Our observations in nude BALB/c mice contrast with those of Wyde et al. (1983) in nude C3H mice. Thus, RSV was cleared from the lungs of nude C3H mice by day 7 of infection. However, virus did not replicate in the lungs of immunologically normal C3H mice. These discrepancies could be related, at least in part, to differences in NK cell activity (Bancroft et al., 1981) or macrophage activation in C3H and BALB/c mice. Extrapulmonary dissemination of RSV was not observed in either mouse strain. Ultimately, the importance of CTL in clearing RSV will be demonstrated by passive transfer of such cells to virus-infected nude BALB/c mice.

During the preparation of this manuscript RSV-specific memory CTL have been demonstrated in the spleens of mice after intranasal infection (R. M. Bangham, M. J. Cannon, D. T. Karzon & B. A. Askonas, unpublished results).

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**REFERENCES**


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