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# Respiratory Syncytial Virus Infection in C57BL/6 Mice: Clearance of Virus from the Lungs with Virus-Specific Cytotoxic T Cells

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**We describe respiratory syncytial virus (RSV)-specific cytotoxic T-cell (CTL) lines and clones developed from the spleens of C57BL/6 and BALB/c mice. Line 7 and clones derived from it were *H-2K*$^b$ restricted, whereas line 12 had both *K*$^b$ and *D*$^b$ components. Both lines, and all the clones except one, could lyse targets infected with either strain A or strain B RSV. Line 7 or 7-11E1 cells ($8 \times 10^6$ to $10 \times 10^6$) given intravenously cleared RSV from the lungs of infected mice. There was no morbidity or mortality in any of the infected mice whether or not they received T cells. The C57BL/6 mouse is a useful model system in which to study the role of the CTL response in protective immunity to RSV. CTL lines and clones can mediate clearance of RSV from the lungs of normal mice without producing any associated morbidity.**

The respiratory syncytial virus (RSV), a pneumovirus within the family *Paramyxoviridae*, is the leading cause of lower respiratory tract disease in infants and young children. Although prevention of RSV infection is a desirable health goal, empirical vaccine approaches have not been successful (11). A formalin-killed vaccine tested in the 1960s did not protect but enhanced the severity of the disease when the infants acquired RSV infection (13).

The mechanisms of protective immunity to RSV are not well understood. Humans are easily reinfected with the virus, but severity of infection and involvement of the lungs diminishes with each recurrence (7). Achieving complete protection from infection may thus not be feasible. Infection with RSV, unlike infection with other members of the family *Paramyxoviridae*, does not confer long-lasting solid immunity upon the host, but reinfections may strengthen immune mechanisms that protect the lower respiratory tract after infancy. Antigenic variation does not seem to account for this failure to develop long-lasting protective immunity, as is the case with the influenza virus. Since involvement of the lower respiratory tract accounts for much of the morbidity and mortality of RSV infection in infancy, understanding the mechanisms that limit viral replication or that might enhance pathology in the lower respiratory tract is of relevance to vaccine design.

Studies with animal models have shed some light on possible factors involved in protective immunity to the virus. In both primate and rodent models, lung infection can be prevented by administration of high titers of neutralizing antibodies (10). In addition, mice and cotton rats can be protected by immunization with either purified glycoproteins or vaccinia virus vectors expressing RSV genes (16, 23, 24). Immunization with the fusion protein, F, appears to be more effective than immunization with the attachment glycoprotein, G (16). There is also evidence that some protection is seen after immunization with vaccinia virus vectors expressing internal virus proteins, possibly mediated by cytotoxic T cells (CTL) (14). Immunization of cotton rats with formalin-killed RSV vaccines reproduces some of the pathological changes observed during the clinical trials with infants (18).

Children with defects in cell-mediated immunity have difficulty clearing the virus and are more susceptible to severe lung infection, thus implicating the T cell as an important immune component in the control of RSV (8). CTL have been shown to have a major immunoprotective role in many experimental viral infections (25) but can also contribute to immunopathology (26); its precise role thus needs to be defined for each infection. If a major histocompatibility complex (MHC) class I-restricted CTL response is necessary for optimal protection against RSV lower respiratory tract disease, then a killed-virus vaccine or a subunit vaccine might not work optimally. Live virus infection is much more effective at inducing such a CTL response (15). This is particularly relevant for RSV, for which there appears to be a delicate balance between immunoprotection and immunopathology both in children (13) and in the rodent models (4, 18).

This article reports the generation and characterization of RSV-specific, MHC class I-restricted CTL in both C57BL/6 and BALB/c mice. We show that cross-reactive CTL lines and clones specific for RSV could be readily generated and that they completely cleared RSV from the lungs without producing any increase in morbidity and with no mortality.

**Infection of mice with RSV.** RSV from the Long (subgroup A) and 18537 (subgroup B) strains were used. Virus stocks were grown on HEp-2 cells cultured in minimal essential medium (MEM) supplemented with 5% fetal bovine serum, 2 mM glutamine, penicillin, and streptomycin. Pools of virus were kept frozen at −80°C. Serum-free virus was prepared in MEM supplemented with 0.1% gelatin. Virus was titered on 24-well plates seeded with a semiconfluent layer of HEp-2 cells. Viral titers were calculated from the Reed and Muench (19) formula and expressed as the 50% tissue culture infectious dose ($TCID_{50}$). C57BL/6 and BALB/c male mice inoculated intranasally with $1 \times 10^6$ to $2 \times 10^6$ $TCID_{50}$s of Long strain (subgroup A) RSV developed infection that could be readily detected in lung homogenates. Peak titer in C57BL/6 mice 5 to 6 days after inoculation was 4.7 ± 0.17 ($\log_{10}$ $TCID_{50}$ RSV per gram of lung tissue ± 95% confidence interval, based on average of titers in 17 mice). There was no

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mortality or any signs of illness associated with RSV infection in the mice. Two weeks after infection, virus could no longer be detected in the lungs.

**Generation of CTL.** Mice recovering from infection had RSV-specific CTL in the spleen, but to reproducibly detect high levels of CTL activity in all mice, a second injection of $4 \times 10^6$ $TCID_{50}$s of live RSV grown in serum-free medium was administered intraperitoneally 3 to 4 weeks later. To generate CTL, 4 weeks after the last RSV inoculation $2 \times 10^7$ spleen cells were restimulated in vitro with $2 \times 10^6$ RSV-infected thioglycollate-induced peritoneal exudate cells (PECs). The PECs were adhered to the base of an upright T25 tissue culture flask before infection with RSV at a multiplicity of infection of 2. After 5 to 7 days of in vitro stimulation, CTL activity was detected by a standard $^{51}Cr$ release assay. Target cells used in the experiments were as follows: (i) thioglycollate-induced PECs, either uninfected or infected with RSV by incubating them overnight with live RSV at a multiplicity of infection of 2, and (ii) BCH4 fibroblasts which are chronically infected with Long strain RSV (6) or its parent BALB line. Targets were labelled with $^{51}Cr$ in the presence of 0.3 M sucrose before being incubated with the effectors at 37°C for a 6-h $^{51}Cr$ release assay. Percent specific $^{51}Cr$ release was calculated as (test cpm-background cpm)/(total cpm-background cpm), where cpm is counts per minute. Background release was always less than 20% of the total, which was determined from targets lysed with 2% Nonidet P-40.

Initial experiments showed that BALB/c spleen cells could readily lyse syngeneic RSV-infected PECs and the BCH4 fibroblasts but not the uninfected parent BALB line. They did not lyse RSV-infected C57BL/6 PEC targets. The C57BL/6 splenocytes could readily lyse syngeneic RSV-infected PECs but not BALB/c PECs. $^{51}Cr$ release from uninfected targets was always less than 5%.

**CTL lines and clones.** To establish CTL lines, spleen cell preparations with CTL activity were restimulated weekly with irradiated (2,000 rad) RSV-infected PECs. An essentially concanavalin A-free rat spleen cell supernatant (20) was used as a source of interleukin-2 (IL-2); it produced 50% maximal proliferation of CTLL cells (9) at a 1% concentration and had 30 U of IL-2 per ml if calibrated against recombinant human IL-2. The concanavalin A supernatant was used at a 10% concentration for growth of the T-cell lines and at a 20% concentration for T-cell cloning. After the third weekly in vitro stimulation, exponentially growing numbers of cells with very high CTL activity were visible. CTL clones were established by limiting dilution in 96-well flat-bottom plates. CTL were usually seeded at 0.3, 1, 3, 10, 30, and 100 cells per well. Each well contained $2 \times 10^4$ RSV-infected, irradiated PECs, $5 \times 10^5$ normal, irradiated spleen cells as fillers, and the CTL. The total volume was 0.2 ml in T-DMEM (Dulbecco's MEM with 10% heat-inactivated fetal bovine serum [HyClone]; 2 mM glutamine; $5 \times 10^{-5}$ M 2-mercaptoethanol; 0.05 mg of gentamicin per ml; 7.2 mM sodium pyruvate; 0.02 mg each of adenosine, guanosine, uridine, and cytidine per ml; 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]; 1% MEM vitamins, 2% MEM nonessential amino acids, 1% MEM essential amino acids with 20% concanavalin A rat spleen supernatant). Wells with growing CTL clones were usually evident after 10 days in culture. These were expanded into 24-well plates with proportionally increased numbers of stimulators and fillers. At this point, they were screened for CTL activity and expanded further into upright T25 flasks. Clones were maintained like the lines by weekly antigenic stimulations. Growth of these CTL lines and clones was strictly IL-2 and antigen dependent. Removal of IL-2 led to almost immediate cessation of growth. Removal of RSV led to 75% diminished growth during the first week, with no growth thereafter even in the presence of IL-2 supernatant. When the C57BL/6 lines and clones were seeded at $2 \times 10^5$ per upright T25 flask, a 25- to 50-fold increase in cell number was evident within 5 to 6 days. Growth of the BALB/c lines and clones was never this vigorous, so the C57BL/6-derived lines and clones were chosen for further characterization.

TABLE 1. MHC restriction pattern of RSV-specific CTL lines and clones

| T-cell effector[a] | % Specific $^{51}Cr$ release from RSV-infected targets[b] | | | |
|---|---|---|---|---|
| | C57BL/6 ($K^bD^b$)[c] | B10.HTG ($K^dD^b$) | B10.MBR ($K^bD^q$) | B6.CH-$2^{bm1}$ ($K^{bm1}D^b$) |
| 2D9 | 2.2[d] | 45.6 | 1.9 | ND |
| Line 7 | 37.2 | 1.0 | 32.3 | 0.0 |
| 7-11E1 | 40.2 | 2.1 | 36.4 | 0.0 |
| 7-13C4 | 36.5 | 3.9 | 29.0 | 0.2 |
| Line 12 | 63.0 | 20.0 | 18.7 | 44.7 |
| 12-10F9 | 67.6 | 3.0 | 50.7 | 3.3 |
| 12-3H1 | 59.6 | 0.3 | 38.3 | 5.8 |
| 12-3C7 | 54.7 | 46.0 | 9.2 | 38.8 |
| 12-3E11 | 42.6 | 14.4 | ND | 44.0 |
| 12-3D1 | 36.2 | 39.6 | 5.4 | 34.6 |

[a] All the T-cell lines and clones, except for 2D9, were derived from C57BL/6 (*H-2*$^b$) mice; 2D9 was derived from a BALB/c (*H-2*$^d$) mouse. The T-cell lines and clones were always used 4 to 6 days after the most recent in vitro stimulation.

[b] Targets are PECs from the indicated mouse strain. They were infected with Long strain RSV $^{51}Cr$ labelled as described in the text. $^{51}Cr$ release was measured in a 6-h assay. Effector-to-target ratios were 20:1 for the line derivatives and 2D9 and 10:1 for the others. There were $10^4$ target cells per well. ND, not determined.

[c] *H-2* haplotype at the *K* and *D* class I loci.

[d] Each result shown is the average of triplicate determinations. Percent $^{51}Cr$ release from uninfected targets was always less than 5% and is not shown.

Line 7 was derived from a C57BL/6 mouse, and 7-11E1 and 7-13C4 were derived by cloning line 7 by limiting dilution. They grew from wells initially seeded at 20 cells per well, with 21 of 48 wells positive for growth, thus giving a cloning efficiency of 2.9%. After expansion, the clones were maintained by weekly stimulations like the parent line 7. Line 12 was also of C57BL/6 origin. 12-10F9 was cloned at 10 cells per well, and 35 of 96 wells had growth giving a 4.5% cloning efficiency. Clones 12-3H1, 12-3C7, 12-3E11, and 12-3D1 grew with 5 to 7% cloning efficiency from wells initially seeded with three line 12 cells per well. All clones expanded from the microwells maintained the growth dependence on RSV and IL-2 of the parent lines. The lytic activity of the lines and clones against RSV-infected targets remained greater than 3 standard deviations above background down to an effector-to-target ratio of 0.3:1.

Table 1 shows the MHC restriction pattern of the CTL lines and clones. Line 7 as well as clones 7-11E1 and 7-13C4 are clearly *H-2K*$^b$ restricted. Line 12 had both *K*- and *D*-restricted components. The clones derived from line 12 were either $K^b$ or $D^b$ restricted. None of the $K^b$-restricted clones could lyse RSV-infected targets that contained the $K^{bm1}$ mutation, which became a simple criterion for determining the MHC restriction pattern of the lines and clones. The $K^{bm3}$ mutation did not affect CTL recognition of the RSV-infected targets. The MHC restriction pattern of an additional 21 clones derived from line 12 was examined

directly after expansion from 96- to 24-well plates: 14 of 21 were $K^b$ restricted, and 7 of 21 were $D^b$ restricted. The results shown in Table 1 are from clones that had been expanded in T25 flasks and maintained in culture longer than 4 weeks. The table also shows 2D9, a BALB/c-derived CTL clone. 2D9 was also lytic for BCH4 targets and for RSV-infected $K^d$ PECs. It did not lyse uninfected targets. Growth of 2D9 was always very sluggish, and after 6 weeks in culture, 2D9 died.

In the original description of CTL to RSV in mice (1), $K^b$ was found to be a nonresponder allele, whereas the clones described here were clearly restricted to either $K^b$ or $D^b$. Our lines and clones were not tested until they had been in culture for longer than 1 month, so it is possible that some in vitro selection had occurred, as has been previously described with influenza virus (22). That target cells from $K^{bm1}$ mutant mice were not recognized by the $K^b$-restricted CTL is entirely consistent with the view that the $K^{bm1}$ mutation affects amino acids of the K molecule directly involved in peptide binding (2).

The RSV subgroup specificity of the CTL lines and clones was explored. Lines 7 and 12 recognized both subgroup A (Long strain) and subgroup B (18537) of RSV. These two strains differ primarily in the sequence of the G surface glycoprotein (12). 7-11E1 had the same cross-reactivity pattern as line 7, from which it was derived. Of the six examined clones derived from line 12, five had the same cross-reactivity pattern as the parent line, whereas 12-3E11 did not lyse the 18537-infected target. 12-3E11 and 12-3C7 are $H\text{-}2D^b$ restricted, and the other clones tested were $H\text{-}2K^b$ restricted. These CTL thus appear to cross-react with both major subgroups of RSV, although there is evidence of a subgroup-specific component in the repertoire. These CTL were derived from mice immunized with Long strain RSV and maintained in culture with the same strain of RSV, suggesting that the high degree of cross-reactivity seen is intrinsic to the CTL repertoire against Long strain RSV in C57BL/6 mice. These T cells are thus presumably directed at conserved sequences of the RSV genome. Such subgroup cross-reactivity had been found previously with BALB/c mouse CTL (17).

**In vivo effect of CTL lines and clones.** In order to investigate the potential role of these T cells on reducing virus titers in the lungs, we chose to inject the CTL into normal C57BL/6 mice 24 h after infection with RSV. This is a time when the titer of RSV in the lungs is at a minimum (21). We reasoned that elimination of the first wave of cells infected with virus would lead to a much lower titer on days 5 to 6, the time at which maximal titer is detected (21). In Table 2 are the results of three separate experiments showing that line 7 as well as clone 7-11E1 was able to eradicate virus from the lungs of RSV-infected mice. In 9 of 13 mice, RSV could not be detected in the lungs. In the other four mice, the $\log_{10}$ $TCID_{50}$ RSV titer was 1 to 3 log units lower. The mice that broke through with a small amount of virus in the lungs were the ones in which we had noted that not all the infusion had entered the vein smoothly. These mice most likely received fewer CTL, which suggests that $8 \times 10^6$ to $10 \times 10^6$ CTL is close to the number needed for a maximal beneficial effect in this system. These experiments also show that exogenous IL-2 need not be added in order for the protective effect to be observed. Line 7 cells injected 5 days after infection (rather than 1) did not result in a clear-cut reduction of the titer detected on day 6 (results not shown). We presume that 24 h was not long enough for the protective effect to be seen, especially at a time when the lung RSV titer is at its peak. Mice used for all these experiments remained without overt signs of illness whether or not they received T cells after the RSV infection. We specifically looked for signs such as respiratory distress, lethargy, and ruffled fur but found none.

TABLE 2. Eradication of lung RSV in vivo by CTL lines and clones[a]

| Expt no. | CTL transferred[b] | Use of IL-2[c] | Lung RSV titer[d] per mouse | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| 1 | None | Yes | 5.0 | 4.7 | | |
| | Line 7 | Yes | ≤2.2 | ≤2.2 | 3.7 | |
| 2 | None | Yes | 4.9 | 4.7 | 5.2 | |
| | 7-11E1 | Yes | ≤2.2 | ≤2.2 | ≤2.2 | |
| 3 | None | Yes | 5.4 | 5.0 | 4.4 | 4.7 |
| | 7-11E1 | Yes | ≤2.2 | ≤2.2 | 2.4 | |
| | 7-11E1 | No | ≤2.2 | ≤2.2 | 2.4 | 3.7 |

[a] C57BL/6 male mice were anesthetized with pentobarbital intraperitoneally and infected intranasally with $10^6$ $TCID_{50}$s of Long strain RSV.

[b] Twenty-four hours after infection with RSV, each mouse received either $8 \times 10^6$ to $10 \times 10^6$ viable T cells intravenously or control medium.

[c] Indicated groups of mice received 0.1 ml of MLA-144 (3) supernatant containing 1,000 U of IL-2. The IL-2 was administered together with the T cells intravenously and again 24 h later intraperitoneally. Mice not receiving IL-2 received control medium.

[d] Five days after infection, the mice were sacrificed, and their lungs were removed, homogenized, and titered on HEp-2 cells. Each value represents the $\log_{10}$ $TCID_{50}$ RSV per gram of lung tissue for an individual mouse. For values less than 2.2, no virus was detected. The lower limit of detection of the assay is $10^{2.2}$ $TCID_{50}$s per g of lung tissue.

These experiments clearly show that CTL can clear RSV from the lungs of mice. The cell transfer nature of these experiments establishes that CTL can mediate clearance of this virus but not that they are necessarily required; CD-8 depletion experiments would provide direct evidence for this. The mechanisms by which CTL exert their protective effect are not completely understood. We believe that since CTL can lyse virus-infected cells before mature virus particles are produced, they can prevent a large number of virus particles from being released. In addition, CTL may release lymphokines such as gamma interferon that might limit viral replication.

Previously, Cannon et al. (5) had reported that RSV-primed CD8-positive spleen T cells could clear RSV from nude mice, which are unable to clear the virus on their own. Subsequently, they reported that BALB/c CTL lines and clones could clear RSV from normal as well as irradiated mice, but with a major increase in morbidity, mortality, and lung pathology (4). We did not observe any morbidity or mortality in our experiments. There are several differences between their system and ours. First, their CTL were administered 3 h postinfection with RSV rather than 24 h in our system. Second, inhaled ether was used for anesthesia, whereas we used pentobarbital intraperitoneally. Third, we used RSV of the Long strain, rather than the A2 strain. Fourth, we inoculated our mice with 10 times more virus than Cannon et al., yet we achieved similar titers in the lungs. Finally, female BALB/c mice were used for their experiments, whereas male C57BL/6 were used for ours. These two systems are thus not directly comparable. Several biological factors might affect the degree of immunopathology associated with the CTL-mediated antiviral effect. BALB/c mice may be more susceptible to the spread of RSV and thus develop morbidity more readily from the effects of widespread lysis of infected cells. In addition, MHC genes, by selecting the viral epitopes accessible to CTL, could

influence the type and magnitude of the CTL target cell interaction. CTL against a particular epitope that is expressed at high levels in the lungs might be more harmful than CTL against a less-exposed epitope. To address these issues, we are planning experiments that will directly compare *H-2*$^b$ and *H-2*$^d$ CTL clones in RSV-infected $F_1$ mice. The fact that Cannon et al. (5) could efficiently clear RSV from nude mice by injecting RSV-primed (fresh, not cultured) spleen cells without any increase in morbidity suggests that some of the observed detrimental effects can be an artifact of CTL growth in vitro.

These experiments establish the C57BL/6 mouse as a model system with which to explore the biology and fine specificity of the CTL response to RSV. They also provide the first evidence that MHC class I-restricted CTL lines and clones can protect the lungs from RSV infection without producing an increase in morbidity or mortality. This is thus a useful system in which to study this mechanism of protective immunity against RSV. Our demonstration that CTL can clear RSV from C57BL/6 mice, the previous experience with BALB/c mice (5), and the observations that humans with deficits in cell-mediated immunity have difficulty clearing the virus suggest that MHC class I-restricted CTL play an important role in clearing RSV from the lungs. Whether induction of an MHC class I-restricted CTL response would enhance the protective efficacy of an RSV vaccine for human infants will probably not be known until results of appropriate studies are available.

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