Brief Definitive Report

CYTOTOXIC T CELLS CLEAR VIRUS BUT AUGMENT LUNG PATHOLOGY IN MICE INFECTED WITH RESPIRATORY SYNCYTIAL VIRUS

By M. J. CANNON, P. J. M. OPENSHAW, and B. A. ASKONAS

From the Division of Immunology, National Institute for Medical Research, London NW7 1AA, United Kingdom

Respiratory syncytial virus (RSV) is an ubiquitous paramyxovirus belonging to the genus *Pneumovirus* (1). Natural human RSV infections occur in winter outbreaks, usually resulting in common cold syndrome. Infants are especially prone to lower respiratory tract infection, which is the most common single cause of hospitalization of this age group in industrial countries. Vaccination with formaldehyde-inactivated RSV induces neutralizing serum antibodies, but fails to protect against natural infection; moreover, infected vaccinees suffer exacerbated lung disease. Similarly vaccinated cotton rats show enhanced pathological changes in the lungs (2), as do mice primed with recombinant vaccinia viruses expressing single RSV proteins (3). These methods of priming induce both humoral and cellular immune responses to RSV (2–5). Passive transfer of mAbs has not been shown to enhance pathology in RSV-infected mice, and some antibodies protect against pulmonary disease (6, 7). Cell-mediated immunity may therefore play a role in the pathogenesis of RSV-induced disease. Although polyclonal memory T cells can clear persistent RSV infection in immunodeficient mice (8), the role of T cell subpopulations in immunopathology merits examination.

In this study, we use bronchoalveolar lavage (BAL) to monitor pulmonary disease in RSV-infected mice, and show enhanced pathology associated with accelerated clearance of lung virus after intravenous transfer of a cytotoxic RSV-specific T cell line and a CTL clone.

Materials and Methods

Virus Infection. The human A2 strain of RSV was grown and assayed for infectivity in HEp-2 cells (9, 10). Etherized female BALB/c mice (SPF bred at NIMR) were infected intranasally with 2×10^5 plaque-forming units (PFU) of RSV.

T Cell Line and Clone. An RSV-specific T cell line (MJC-A2) was derived from BALB/c mice primed by infection with RSV. Spleen cells were stimulated with antigen every 7 d in 25-cm^2 plastic flasks (Nunc, Roskilde, Denmark; Gibco-BRL, Paisley, Scotland). 3×10^6 T cells were cultured in 20 ml RPMI/10% FCS with 2×10^7 syngeneic X-irradiated (3,000 rad) spleen cells infected with RSV at a multiplicity of infection (moi) of 0.1 PFU per cell.

Address correspondence to B. A. Askonas, Division of Immunology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom. M. J. Cannon's present address is the Immunology Department, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037.

After 4–5 cycles, the cultures were supplemented every 3–4 d with 10% crude IL-2, provided by 48-h supernatants from Con A–stimulated rat spleen cell cultures. MJC-A2 T cells were used in adoptive transfer experiments after 15–20 weekly passages. CTL clone E8a was isolated 7 d after the first in vitro antigenic stimulation of spleen cells from primed BALB/c mice. T cells were distributed in 96-well plates with syngeneic RSV-infected X-irradiated spleen cells (5×10^5/well). Cultures were supplemented with 10% crude IL-2 (see above) at day 0 and 7. CTL clone E8a was maintained as described above.

T Cell Transfers. BALB/c mice were infected intranasally with RSV A2, and within 3 h of infection, CTL were transferred intravenously in 200 μl HBSS. In some experiments, mice were irradiated (500 rad, ^{60}Co source) 24 h before infection with RSV and/or transfer of CTL.

Bronchoalveolar Lavage (BAL). Mice were killed with and exsanguinated via the femoral vessels. The thorax was opened, and 1.2-mm Portex tubing (Portex, Hythe, England) was introduced to the trachea at the cricothyroid membrane. 1 ml of 12 mM lidocaine hydrochloride (Astra Pharmaceuticals, King's Langley, England) in PBS was washed in and out six times over a 1–2-min period to promote elution of adherent cells. 2 ml of hemolytic HBSS (BSS minus NaCl, plus NH_4Cl, and 0.5% gelatin) was added to the eluate for 5 min, and 200 μl was removed for spectrophotometric estimation of hemoglobin (Hb) concentration at 405 nM. Background absorption at 492 nM was deducted. Hb concentration was expressed as μg/ml of BAL fluid. Normal BALB/c blood was used as a calibration standard, and assumed to contain 150 mg/ml Hb. The remaining cells were washed, cytospun onto slides, fixed, and Giemsa stained. Differential counts of 300–500 cells were made by oil immersion light microscopy. Cytotoxicity (see below) was assayed after removal of plastic adherent cells for 1 h at 37°C.

Cytotoxicity Assays. The ^{51}Cr-release assay for RSV-specific CTL has been described (9). Target cells were P815 cells or K^d-transfected L cells (L-K^d), either uninfected, or infected for 24 h with RSV A2 strain at an moi of 1–2. L-K^d cells were kindly provided by Dr. J. Maryanski, Ludwig Institute, Lausanne, Switzerland. BCH4 cells are a BALB/c fibroblast line persistently infected with Long strain RSV (11).

Results

Characterization of RSV-specific T Cell Line MJC-A2 and CTL Clone E8a. MJC-A2 T cells were 80% Lyt-2$^+$, but also contained 20% L3T4$^+$ cells; CTL clone E8a was exclusively Lyt-2$^+$ and L3T4$^-$. Both the line and clone were dependent on exogenous IL-2 and RSV for growth and lysed P815 cells infected with RSV. Clone E8a was K^d restricted (71% specific lysis of RSV-infected L-K^d cells), but grew much slower than the line. Helper T cell function was examined by assaying release of IL-2 (5) and IL-3 (tested on 32D cells) after antigen stimulation. No IL-2 release was detected, and only the A2 line released IL-3 (Lin, Y. L., unpublished results).

Although target lysis was specific for RSV, the RSV protein(s) recognized by the T cell line and CTL clone could not be defined. Neither lysed target cells infected with recombinant vaccinia virus expressing the RSV nucleoprotein, attachment glycoprotein, fusion protein, 1B, 1C, or partial matrix (residues 88–256) gene products (4, 5). It is hoped that antigen specificity will be defined when further VV recombinants become available.

Transfer of CTL Augments Disease in Infected BALB/c Mice. Since irradiated mice can be persistently infected with RSV (8), we examined whether such infection could be cleared by transfer of CTL. RSV-infected recipients of the T cell line became ill within 2–3 d of cell transfer; they lost weight, developed ruffled fur, became tachypnoeic, and cyanosed. High mortality resulted, and only one out of five mice survived to day 5 (Fig. 1). RSV infection alone produced no signs of distress, with a mean

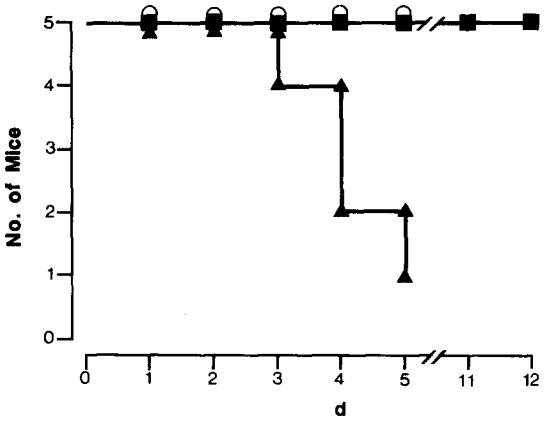

FIGURE 1. Survival of γ-irradiated (500 rad) BALB/c mice after RSV infection and/or transfer of MJC-A2 T cells at day 0. Mice were irradiated on day -1. Treatments were (○) 10^5 pfu RSV i.n.,; (■) 8×10^6 MJC-A2 Tc i.v.; (▲) 10^5 pfu RSV i.n., and 8×10^6 MJC-A2 CTL i.v. RSV-infected mice were killed on day 5 for lung RSV assays. Mean RSV titre (log_{10} pfu/pair of lungs): (○) 4.7 and (▲) <1.8 (one survivor at day 5). Mice given MJC-A2 cells alone showed no signs of illness.

lung RSV titer of 4.7 log_{10} PFU at day 5. Uninfected irradiated mice receiving only MJC-A2 cells showed no signs of illness.

Since the single survivor of the RSV-infected group that received MJC-A2 T cells was clear of lung virus on day 5, we examined whether transfer of reduced numbers of CTL could clear a persistent RSV infection without leading to lethal respiratory disease. In irradiated RSV-infected mice given 10^6 cells, mean lung RSV titer was greatly reduced by day 5 (2.0 log_{10} PFU compared with 4.0 log_{10} PFU in mice not given T cells), and on day 10, all mice given T cells were clear of RSV while controls had 3.5 log_{10} PFU in the lungs. Transfer of 3×10^5 MJC-A2 cells resulted in clearance by day 10, but gave a much smaller, though significant ($p < 0.02$), reduction in lung RSV on day 5, indicating less rapid virus clearance. All mice survived to day 10, with progressively increasing signs of respiratory distress in infected mice as the number of transferred CTL was increased. Uninfected irradiated mice given 3×10^6 cells all appeared well.

Infection of normal BALB/c mice results in peak lung RSV titers on day 4–6;

TABLE I

Transfer of MJC-A2 Cells and CTL Clone to RSV-infected Mice

RSV (i.n.)	Number of cells (i.v. × 10^{-6})	Exp. 1: T line MJC-A2			Exp. 2: CTL clone E8a		
		Lung virus	BAL		Lung virus	BAL	
			PMN	Hb		PMN	Hb
		log_{10} PFU	*%*	*μg/ml*	*log_{10} PFU*	*%*	*μg/ml*
+	0	3.9(3.4–4.2)	2.8(0–12)	1.7(0–8)	3.9(3.7–4.1)	5.6(1–17)	8.9(2–33)
+	1	<1.8(all)	8.7(0–68)	133(14–930)	ND	ND	ND
+	3	<1.8(all)	25.1(12–40)	280(230–310)	<1.8(all)	11.4(4–60)	278(80–1200)
0	3	-	1.3(0–3)	<1(all)	-	1.3(0–3)	3.2(0–54)

Geometric mean and range (in parentheses) of values of various parameters for groups of normal BALB/c mice subjected to infection and/or CTL transfer. (Exp. 1) Mice were given MJC-A2 cells and groups subjected to bronchoalveolar lavage (BAL) and virus titration at day 5. Two out of five infected mice given 3×10^6 CTL cells died before day 5, and the other three showed respiratory distress. (Exp. 2) Mice were injected with CTL clone E8a; one out of six infected mice given 3×10^6 cells died before day 5, and others in this group appeared very ill. None of the control mice (infected or CTL alone) showed respiratory distress.

thereafter, the virus is rapidly cleared (12). We therefore examined virus titer and pathology at day 5 in normal mice, a time point by which a primary CTL response is not detectable in the lungs (13). As shown in Table I, transfer of 10^6 or 3×10^6 MJC-A2 cells to RSV-infected immunocompetent mice efficiently cleared lung RSV by day 5, but both groups again showed signs of respiratory distress. Of the infected mice receiving 3×10^6 MJC-A2 T cells, two out of five were dead by day 5. Pathological effects were monitored by BAL. Although lung hemorrhage was statistically significant in groups of infected mice given either 10^6 or 3×10^6 T cells ($p < 0.01$ and $p < 0.05$, respectively; Mann-Whitney test) compared with those only infected with RSV, the groups do not show statistically significant differences in polymorphonuclear (PMN) cell counts. There is considerable individual variation in PMN efflux into BAL, but two out of five and two out of three surviving host mice have exceptionally high PMN counts (30–60%) after transfer of 10^6 and 3×10^6 A2 T cells, respectively.

Despite proven pulmonary infection, the control mice not given CTL appeared well and had low Hb levels and PMN counts in BAL, and uninfected mice receiving MJC-A2 T cells similarly showed no pathology. Histological examination of the lungs of infected Tc recipient mice showed increased peribronchiolar infiltration of lymphocytes, monocytes, and PMNs compared with those infected with RSV alone. In addition, areas of alveolar consolidation with hemorrhage were seen (not shown).

Transfer of CTL clone E8a also led to increased pulmonary disease in immunocompetent RSV-infected mice (Table I, Exp. 2). 3×10^6 E8a cells resulted in clearance of RSV from the lungs of surviving RSV-infected mice by day 5 and a striking pulmonary hemorrhage ($p < 0.01$). Again, PMN counts >20% were only seen in infected mice injected with CTL, but group differences were not statistically significant. Transfer of influenza NP-specific CTL clone BA4 (14) into RSV-infected mice did not enhance lung pathology (not shown).

Can RSV-specific CTL Be Detected in BAL Cells? BAL cells were recovered from the lungs 4 d after RSV infection with or without T cell transfer. Nonadherent BAL cells from uninfected mice receiving the T cell line or from infected nonrecipient mice showed no cytotoxicity, whereas, the infected T cell recipients showed detectable but low cytotoxicity (BCH4 cell lysis of 11.3 ± 0.7%, compared with 3.8 ± 0.6% of uninfected BALB/c fibroblasts at K/T of 50:1; not illustrated). RSV-specific CTL could not be found in the spleens of any of these groups of mice. CTL can therefore only be eluted from the lungs of T cell–recipient mice with pulmonary RSV infection.

Discussion

Our results show that RSV-specific CTL are able to eliminate virus from the lungs of RSV-infected mice, and that the rate of virus clearance depends on the number of cells injected. When large numbers of CTL are injected intravenously (10^6 or more), virus clearance is associated with augmented and sometimes lethal pulmonary pathology. Similar results are obtained with cloned CTL and a T cell line, despite the presence of some $L3T4^+$ cells in cytotoxic line MJC-A2. Further experiments with the clone were limited by its slow and erratic growth rate in vitro.

Virus-specific CTL have been shown to be capable of mediating both virus clearance and immunopathology in mice infected with lymphocytic choriomenigitis virus

(15). Our results with RSV-infected mice contrast with previous experiments from this laboratory in which 6–10 × 10^6 influenza-specific cloned CTL greatly reduced influenza virus titres in lung and trachea of influenza-infected mice (14) and led to early reduction of histopathological changes in the CTL recipients (Mackenzie, C., P. M. Taylor, and B. A. Askonas, manuscript in preparation). The augmented hemorrhagic lung disease, which we now describe, has no parallel in influenza infected mice.

One possible reason for these contrasting findings is the different anatomical distribution of the virus infection. Fluoresceinated antibody to RSV binds to cells in the bronchial and bronchiolar epithelium and alveolar cells in the lungs of RSV-infected children, Cebus monkeys, and mice (1, 12). It is suggested that alveolar spread of viral antigen occurs from the replication site in bronchiolar epithelium. By contrast, influenza virus is not found in the alveoli (16). Inflammatory edema and cellular infiltration is more likely to cause disease if it occurs in small airways, since small changes in caliber will produce large changes in airway resistance. Necrosis of cells in small airways and alveoli is likely to produce hemorrhage, since blood and air are separated by very small distances in this zone of the lung.

Patients with RSV-induced bronchiolitis have fewer $CD8^+$ lymphocytes in the peripheral blood than those with other forms of RSV infection (17), indicating either that $CD8^+$ cells are depleted or that they are concentrated elsewhere. RSV-specific CTL are undetectable in the peripheral blood of infants with the most severe RSV-induced bronchiolitis (18). Isolation of RSV-specific CTL (13) and large numbers of $CD8^+$ T cells in BAL fluid from the lungs of RSV-infected mice (P. J. M. Openshaw, unpublished results) supports our finding that RSV-specific CTL are concentrated in the lungs during pulmonary infection.

The present results point to the fine balance of protective vs. deleterious cellular effects depending on the level of the CTL response. We now hope to examine whether Th cells are protective or cause pathology similar to that induced by CTL in RSV infection. It is clearly important to explore the function of T cell subpopulations in different virus infections during development of protective vaccines in order to avoid enhancement of subsequent disease.

Summary

We have examined the function of class I MHC-restricted cytotoxic T cells in experimental respiratory syncytial virus (RSV) infection of BALB/c mice by transfer of T cell line MJC-A2 and CTL clone E8a into RSV-infected mice. The T cell line cleared pulmonary RSV infection within 5 d in persistently infected γ-irradiated mice, but caused acute respiratory disease. This was only seen in infected mice and was often lethal after transfer of >3 × 10^6 CTL. Lower numbers of CTL produced less severe disease but still cleared lung RSV, albeit over a longer time course (up to 10 d). Clearance of lung RSV in immunocompetent mice by the T cell line and CTL clone was again accompanied by acute and sometimes lethal respiratory disease. Bronchoalveolar lavage showed severe lung hemorrhage and frequent neutrophil efflux in mice with CTL-augmented disease.

We thank Drs. G. W. Wertz, L. A. Ball, and A. M. Q. King for kindly providing recombinant vaccinia viruses.

Received for publication 6 April 1988 and in revised form 20 June 1988.

References

1. Stott, E. J., and G. Taylor. 1985. Respiratory syncytial virus. *Arch. Virol.* 84:1.
2. Prince, G. A., A. B. Jenson, V. G. Hemming, B. R. Murphy, E. E. Walsh, R. L. Horswood, and R. M. Chanock. 1986. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactivated virus. *J. Virol.* 57:721.
3. Stott, E. J., G. Taylor, L. A. Ball, K. Anderson, K. K. -Y. Young, A. M. Q. King, and G. W. Wertz. 1987. Immune and histological responses in animals vaccinated with recombinant vaccinia viruses that express individual genes of human respiratory syncytial virus. *J. Virol.* 61:3855.
4. Pemberton, R. M., M. J. Cannon, P. J. M. Openshaw, L. A. Ball, G. W. Wertz, and B. A. Askonas. 1987. Cytotoxic T cell specificity for respiratory syncytial virus proteins: fusion protein is an important target antigen. *J. Gen. Virol.* 68:2177.
5. Openshaw, P. J. M., R. M. Pemberton, L. A. Ball, G. W. Wertz, and B. A. Askonas. 1988. Helper T-cell recognition of respiratory syncytial virus in mice. *J. Gen. Virol.* 69:305.
6. Taylor, G., E. J. Stott, M. Bew, B. F. Fernie, P. J. Cote, A. P. Collins, M. Hughes, and J. Jebbett. 1984. Monoclonal antibodies protect against respiratory syncytial virus infection in mice. *Immunology.* 52:137.
7. Prince, G. A., V. G. Hemming, R. L. Horswood, P. A. Baron, and R. M. Chanock. 1987. Effectiveness of topically administered neutralizing antibodies in experimental immunotherapy of respiratory syncytial virus infection in cotton rat. *J. Virol.* 61:1851.
8. Cannon, M. J., E. J. Stott, G. Taylor, and B. A. Askonas. 1987. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. *Immunology.* 62:133.
9. Bangham, C. R. M., M. J. Cannon, D. T. Karzon, and B. A. Askonas. 1985. Cytotoxic T-cell response to respiratory syncytial virus in mice. *J. Virol.* 56:55.
10. Cannon, M. J. 1987. Microplaque immunoperoxidase detection of infectious respiratory syncytial virus in the lungs of infected mice. *J. Virol. Methods.* 16:293.
11. Fernie, B. F., E. C. Ford, and J. L. Gerin. 1981. The development of BALB/c cells persistently infected with respiratory syncytial virus: presence of ribonucleoprotein on the cell surface. *Proc. Soc. Exp. Biol. Med.* 167:83.
12. Taylor, G., E. J. Stott, M. Hughes, and A. P. Collins. 1984. Respiratory syncytial virus infection in mice. *Infect. Immun.* 43:649.
13. Taylor, G., E. J. Stott, and A. J. Hayle. 1985. Cytotoxic lymphocytes in the lungs of mice infected with respiratory syncytial virus. *J. Gen. Virol.* 66:2533.
14. Taylor, P. M., and B. A. Askonas. 1986. Influenza nucleoprotein specific cytotoxic T-cell clones are protective in vivo. *Immunology.* 58:417.
15. Baenziger, J., H. Hengartner, R. M. Zingernagel, and G. A. Cole. 1986. Induction or prevention of immunopathological disease by cloned cytotoxic T cell lines specific for lymphocytic choriomeningitis virus. *Eur. J. Immunol.* 16:387.
16. Wells, M. A., P. Albrecht, and F. A. Ennis. 1981. Recovery from a viral respiratory tract infection. I. Influenza pneumonia in normal and T-deficient mice. *J. Immunol.* 126:1036.
17. Welliver, R. C., T. N. Kaul, M. Sun, and P. L. Ogra. 1984. Defective regulation of immune responses in respiratory syncytial virus infection. *J. Immunol.* 133:1925.
18. Isaacs, D., C. R. M. Bangham, and A. J. McMichael. 1987. Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. *Lancet.* ii:769.