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Cytotoxic T-Cell Response to Respiratory Syncytial Virus in Mice

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The role of the humoral and cellular arms of the immune response in protection against respiratory syncytial virus (RSV) infection and in the pathogenesis of the severe forms of this disease is poorly understood. The recent demonstration that some inbred mouse strains can be infected with RSV has opened the way to a detailed investigation of RSV immunity. We report here the finding of major histocompatibility complex-restricted, RSV-specific memory cytotoxic T cells in the spleens of BALB/c and C57BL mice after intranasal infection; these T cells recognize the Long, A2, and 8/60 (human) strains of RSV. Both K and D locus major histocompatibility complex alleles can restrict the cytotoxic response; however, in the two haplotypes tested, D^d is a low-responder allele and K^b is a nonresponder allele for RSV. UV-inactivated RSV (when given intraperitoneally) can prime mice for development of cytotoxic T cell memory, restimulate cytotoxic T cell cultures in vitro, and form a target for the cytotoxic cells.

Respiratory syncytial virus (RSV) is a widespread paramyxovirus and is the most important viral cause of respiratory disease in infants and young children (10). The immune basis of the difference in susceptibility observed among individuals and of the very limited age range of the severe response is not understood. Further observations which have attracted the interest of immunologists are (i) the annual recurrence of the virus in winter epidemics and the fact that it can repeatedly reinfect the same person (8), although there is extensive serological cross-reactivity between virus isolates (2); (ii) the fact that a Formalin-inactivated RSV vaccine, which underwent clinical trials in the late 1960s, gave no protection against infection despite eliciting neutralizing and complement-fixing antibodies and made the severe forms of the infection still worse (see, e.g., references 9 and 11); and (iii) the intensity of the inflammatory response in bronchiolitis, for which no convincing explanation has emerged (13).

The experimental study of RSV infection and immunity has been hindered by the lack of a suitable model of the disease in inbred animals: the natural host range of the virus is confined to humans and cattle. However, Prince et al. (14) (using neonatal mice), and more recently Taylor et al. (20) (using adult mice) have shown that RSV can infect inbred mice, although the disease produced is not severe. Taylor et al. (submitted for publication) have found major histocompatibility complex (MHC)-restricted, RSV-specific cytotoxic T cells (CTLs), able to lyse a persistently infected cell line, in the lungs of mice until 14 days after acute RSV infection; no CTLs were found in the spleens. We report here that RSV infection of BALB/c and C57BL mice generates H-2 restricted, RSV-specific memory CTLs (26) in the spleens. K^b appears to be a low-responder allele for RSV-specific CTLs. Even after complete inactivation of infectivity by UV irradiation, RSV primes mice effectively for memory CTLs and forms good targets for the CTLs when adsorbed to peritoneal exudate cells (PECs).

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MATERIALS AND METHODS

Virus strains; preparation of stock; plaque assay. Three strains of RSV were used. The Long strain (donated by M. G. Pereira, Central Public Health Laboratory, London, United Kingdom) was purified by three successive passages, and a stock was grown in HEp-2 cells in Dulbecco modified Eagle medium with 10% fetal calf serum, antibiotics (penicillin, 60 μg/ml; streptomycin, 100 μg/ml), and glutamine (300 μg/ml) and harvested after 72 h of incubation at 37°C; aliquots were kept in liquid nitrogen until use. The thawed material had a titer of 2×10^7 PFU/ml. The A2 and 8/60 strains (donated by E. J. Stott, Institute for Research on Animal Diseases, Newbury, Berkshire, U.K.) were used (without plaque purification) to prepare stocks of virus as above: these stocks also had a titer of 2×10^7 PFU/ml. Virus titers were measured by using a plaque assay on HEp-2 cells under a Dulbecco modified Eagle medium overlay containing 0.25% agarose A-37 (Indubiose; Pharmindustrie, Clichy, France), 2% fetal calf serum, antibiotics, and glutamine (as above) and incubated at 33°C for 5 days in 6% CO_2 in six-well (35-mm) plates (Nunclon). The monolayers were fixed in 4% formaldehyde and stained with Giemsa, and plaques were counted under a dissecting microscope. UV-inactivated virus was prepared by exposing 0.6 ml of stock virus in a petri dish (diameter, 30 mm) to a Philips 15-W UV lamp for 2 or 10 min at a distance of 30 cm (radiation intensity, 400 $\mu W/cm^2$).

Infection of mice. BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were bred under specific-pathogen-free conditions at this Institute; B10A.5R (K^bD^d) and D2GD (K^dD^b) were obtained from Olac. BALB/c or C57BL mice at age 4 to 8 weeks were anesthetized with pentobarbitone (30 mg/kg) intraperitoneally (i.p.) and infected intranasally (i.n.) with 2×10^5 PFU of RSV (A2 strain) in 50 μl. To demonstrate lung infection, lungs were removed on day 5 after inoculation, homogenized in ice-cold Hanks balanced salts solution containing 0.218 mol of sucrose per liter (14), antibiotics (as above), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (10 mmol/liter), and centrifuged at 2,000 × *g* for 5 min, and the supernatant was frozen in liquid nitrogen before being

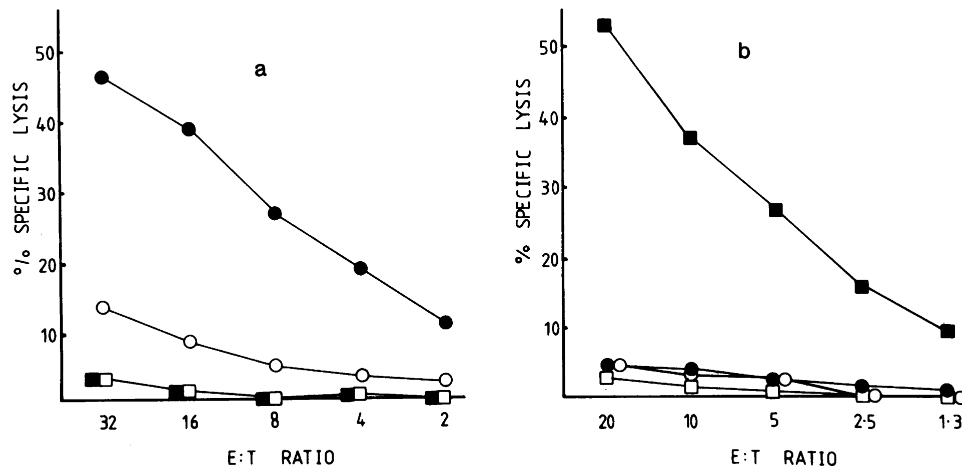

FIG. 1. H-2 restriction and virus specificity of anti-RSV CTLs. Donor mice were primed 2 months previously with infectious A2 strain RSV and restimulated in vitro with the A2 strain. (a) Lysis of RSV-infected (●) and uninfected (○) BALB/c PECs by BALB/c CTLs. C57BL CTLs lysed neither infected (■) nor uninfected (□) BALB/c PECs. (b) Lysis of RSV-infected (■) and uninfected (□) C57BL PECs by C57BL CTLs. BALB/c CTLs lysed neither infected (●) nor uninfected (○) C57BL PEC.

assayed by a plaque assay. Mice primed i.p. with RSV were given 1.6×10^6 PFU of stock virus in 400 μl.

Generation of secondary CTLs in vitro. Spleen cells from donors primed with RSV 2 to 5 months previously were restimulated in vitro with either BCH4 cells irradiated with 2,000 rads or RSV-treated spleen cells. Spleen cells (responders) (1.5×10^7) were incubated with 3×10^6 stimulator cells in 20 ml of RPMI 1640–10% fetal calf serum (RPMI-10) with 5 mmol of 2-mercaptoethanol per liter at 37°C for 5 to 7 days before the cytotoxicity assay. Recovery was typically 40 to 70% of responder cells on day 5.

Target cells and cytotoxicity assay. BCH4 cells are a line of BALB/c embryo fibroblasts persistently infected with the Long strain of RSV (6); as controls we used uninfected cells from the same BALB/c line. About 80% of the BCH4 cells express the viral "spike" antigen and large amounts of RSV ribonucleoprotein on the cell surface; the cells do not form syncytia (6). PECs were induced in mice by i.p. injection of 1.5 ml of 3% thioglycolate (no. 0256; Difco Laboratories) and harvested 3 to 4 days later. The presence of RSV on the target cells was demonstrated by immunofluorescence (i) by an indirect method involving the use of individual anti-RSV monoclonal antibodies (anti-F protein, anti-nucleoprotein, or anti-GP84) (donated by B. F. Fernie; see reference 5) as first layer and a fluorescein-conjugated rabbit anti-mouse immunoglobulin as second layer or (ii) by a direct method (with Boots-Celltech Imagen reagent, containing one anti-nucleoprotein and two anti-F monoclonal antibodies, donated by Boots-Celltech Ltd.).

A standard ^{51}Cr release assay was used (27). In brief, BCH4 target cells (and the BALB/c fibroblast control line) were incubated with ^{51}Cr (100 μCi/10^7 cells) for 60 min at 37°C, washed three times in RPMI/10 plus antibiotics and glutamine as described above, and distributed in 96-well plates (Nunc) at 2×10^4 cells (in 100 μl) per well. PECs were incubated with virus at a multiplicity of infection of 0.25 to 1 PFU per cell overnight at 37°C in RPMI-10 in petri dishes (diameter, 35 mm). The cells were then harvested by scraping with a glass rod, incubated with ^{51}Cr as above for 90 min at 37°C, and then washed and distributed as described above. In some experiments the BALB/c fibroblasts were used as target cells; they were infected de novo at 1 PFU/cell and incubated for 48 h in RPMI-10 in petri dishes (diameter, 35 mm) before the cytotoxicity assay. Effector cells were added, and the plate was centrifuged at $150 \times g$ for 30 s before incubation at 37°C for 3 or 4 h. The plate was then centrifuged at $400 \times g$ for 5 min, and 100 μl of the supernatant was counted in a gamma counter. The percent specific lysis (L) was calculated as follows: L = [(sample − background)/(total − background)] × 100, where total = radioactivity released by targets treated with Triton X-100 detergent. Each point is the mean from three replicate wells.

RESULTS

Infection of mice and PECs. Infectious RSV was recovered from the lungs 5 days after i.n. infection of BALB/c mice (median titer, 1.6×10^3 PFU/g) (cf. reference 20). Specific immunofluorescence was detected on nearly 100% of RSV-treated PECs by direct staining (with the Boots-Celltech Imagen reagent) at 2 h after infection; fluorescence detected on indirectly stained cells by using individual monoclonal antibodies (5) was weaker and therefore harder to quantify. RSV-specific lysis of target PECs treated with virus for only 2 h was significant, but the magnitude of the T-cell-mediated lysis was more variable than after overnight treatment, which therefore became the standard assay procedure.

Generation of CTLs: MHC restriction. Figure 1 shows that spleen cells from BALB/c or C57BL mice, primed by i.n. infection with A2 strain RSV, can be stimulated in vitro to generate a strong CTL response. This is not so for normal spleen cells (not illustrated). The CTLs are clearly RSV specific and H-2 restricted and lyse only syngeneic RSV-infected target cells. Thioglycolate-induced PECs treated with infectious RSV (A2 strain) serve as good target cells.

Since in both of the above mouse strains, RSV generates a strong cytotoxicity response, we wished to see whether the K- and D-region molecules served equally well in restricting the response. Target cells were PECs from two congenic mouse strains; D2GD (K^dD^b) targets were efficiently lysed by both BALB/c and C57BL-derived CTLs (Fig. 2a), while PECs from B10A.5R mice (K^bD^d) were lysed by BALB/c CTL effectors, but not at all by C57BL effectors (Fig. 2b). Thus K^b is a nonresponder allele for RSV recognition by C57BL CTLs, and RSV in conjunction with D^d is less well recognized than with K^d. As in other virus systems, there-

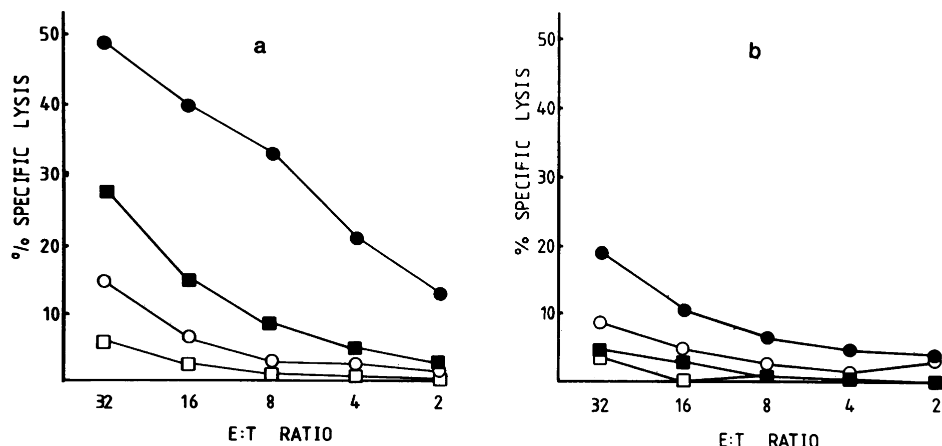

FIG. 2. K and D restriction of RSV-specific CTLs. (a) Lysis of RSV-infected (●) and uninfected (○) D2GD PECs by BALB/c CTLs and infected (■) and uninfected (□) D2GD PECs by C57BL CTLs. (b) Lysis of infected (●) and uninfected (○) B10A.5R PECs by BALB/c CTLs. There was negligible lysis of both infected (■) and uninfected (□) B10A.5R PECs by C57BL effectors.

fore, the RSV CTL response varies with the class I MHC molecule (26).

CTL specificity for three RSV strains. We wished to see whether RSV-specific CTLs would recognize different strains of RSV or whether they were strain specific. CTLs were induced with A2 strain RSV, and targets were PEC treated with A2 or Long strain RSV or the 8/60 strain, a third human strain which is serologically distinct from the A2 and Long strains (with monoclonal antibodies directed against the fusion or attachment proteins; E. J. Stott, personal communication). The most efficient lysis was with A2-infected targets; the Long strain was also recognized, although less well, and PECs treated at the same multiplicity of infection with 8/60 strain virus were poorly recognized (Table 1). The CTLs thus appear to show only poor cross-reaction between different strains of RSV.

UV-inactivated RSV primes mice and induces memory CTLs. Since RSV has a strong fusion protein, it was of interest to determine whether infectivity of the virus is required to prime mice for memory CTLs. Our results show that A2 strain RSV, inactivated by UV (exposure for 2 or 10 min), was still able to prime mice (Fig. 3b) and to restimulate memory CTLs in vitro (Fig. 3), although live virus was more efficient in both instances. Inactivation of the virus was demonstrated by the absence of viral cytopathic effect on HEp-2 cells after 4 days in culture (not illustrated). In these experiments the persistently infected BCH4 cells (and the control BALB/c fibroblast line) were used as target cells, since they are highly susceptible to RSV-specific T-cell-mediated lysis. Usually a two- to threefold higher lysis is obtained than with RSV-treated PECs, and the PECs show some variability in the level of lysis. The BCH4 cells were originally infected with the Long strain of RSV (6), and so their recognition and lysis by CTLs primed and restimulated with A2 strain virus provides further evidence for cross-reactivity at the CTL level between the A2 and Long strains of RSV.

DISCUSSION

Antibody is known to play a part in protection against RSV infection, but may be insufficient to eradicate the virus from the body. Prince et al. (15) showed, by passive transfer of serum or cells from immune hosts, that the immunity to pulmonary reinfection with RSV induced by RSV infection in cotton rats was due to antibodies. However, some animals showed almost complete immunity to RSV despite undetectable antibody titers. Recently, passive transfer of certain monoclonal antibodies (to GP84 and fusion protein) has been shown to confer significant protection to both cotton rats (22) and inbred mice (19). There is epidemiological evidence that colostral antibodies protect against or lessen the severity of RSV infection (4), although neither the occurrence nor the severity of disease has been shown to be related to antibody titers (see reference 13 for review). Ward et al. (23) noted that infants between 6 and 12 months old, unlike older children and adults, were unable to produce antibody against the viral protein VGP95; the significance of this finding to the pathogenesis is not yet known.

It therefore becomes important to study T-cell-mediated responses. The role of T cells in protection or pathogenesis in RSV infection is not clear. Several groups have reported RSV-specific proliferation of peripheral blood lymphocytes in humans after either natural infection or vaccination (see, e.g., reference 16). Fishaut et al. (7) found persistent excretion of RSV (and influenza and parainfluenza 3) in six children with severe congenital immunodeficiencies, both those (three of six) with combined humoral defects and those (three of six) with only cellular immune defects. Sun et al.

TABLE 1. Lysis by BALB/c CTLs of target cells infected with infectious A2, Long, or 8/60 strain RSV, or UV-inactivated RSV (A2 strain)[a]

Expt no.	Virus adsorbed to target cell	% Lysis ± SEM
1	A2	31 ± 1
	A2/UV	33 ± 6
	Long	23 ± 0.5
	—	10 ± 1
2	A2	36 ± 3
	8/60	15 ± 1
	—	2 ± 0.6

[a] Percent lysis of infected syngeneic target cells at an effector/target ratio of 40:1 (experiment 1) or 32:1 (experiment 2). Target cells were PECs treated with virus for 2 h in experiment 1 and BALB/c fibroblasts treated with virus for 48 h in experiment 2. Donor BALB/c mice were primed by i.n. infection with the A2 strain and restimulated in vitro with A2 strain RSV.

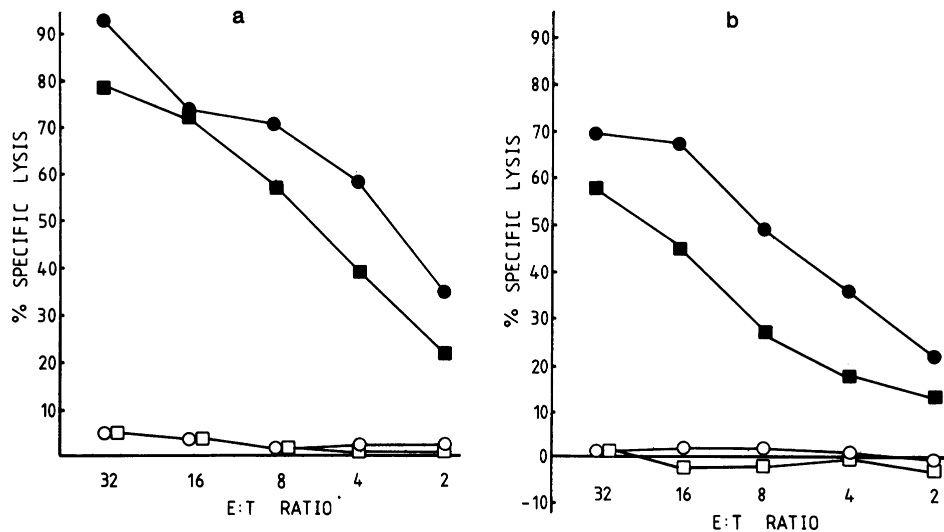

FIG. 3. UV-inactivated RSV can prime for CTLs and restimulate CTLs in vitro. (a) CTLs from BALB/c mice primed i.p. with live A2 strain RSV 3 months earlier. These effectors lysed BCH4 cells whether the effectors were restimulated in vitro by live (●) or UV-inactivated (■) RSV. There was no significant lysis of BALB/c control fibroblasts in either case(○, □). (b) CTLs from BALB/c mice, primed i.p. with UV-inactivated A2 strain RSV 3 months previously. These CTLs again lysed BCH4 cells whether the CTLs were restimulated in vitro by live (●) or UV-inactivated (■) RSV. Uninfected controls (○, □) were not lysed.

(17, 18) demonstrated a longer duration of RSV infection in irradiated than in unirradiated cotton rats, but the cytotoxic activity of lung effector cells was neither histocompatibility restricted nor virus specific. Both athymic (nude) and irradiated C3H mice cleared RSV infection from the lung by 7 days (25), but Prince et al. (14) showed that this mouse strain was one of the least susceptible to the virus.

In this paper we demonstrate the generation of RSV-specific memory CTLs in cultures of spleen cells from BALB/c or C57BL mice primed i.n. with RSV 2 to 5 months previously and restimulated in vitro either with a persistently infected fibroblast line or with virus adsorbed to spleen cells. The CTLs induced with A2 strain virus lysed syngeneic target cells, i.e., fibroblasts persistently infected with Long strain RSV, or PECs whether infected with the Long strain or the A2 strain. The serologically distinct 8/60 strain was less well recognized. The MHC restriction and virus specificity suggest that these effector T cells are classical CTLs (26). The apparent failure of CTLs to recognize RSV in conjunction with the K^b allele is similar to isolated gaps in the antigenic repertoire of CTLs that have been described with other viruses (26). In particular, Doherty et al. (3) found K^b to be a nonresponder allele in the CTL response against influenza A.

The RSV antigens recognized by the CTLs remain to be defined. The surface expression of large amounts of RSV ribonucleoprotein, in addition to the "spike" protein on a high proportion of the persistently infected fibroblasts used here as target cells (6), raises the interesting possibility that an internal viral antigen is recognized by these CTLs; there is evidence for an analogous phenomenon in influenza (21).

Virus inactivated with UV light and given i.p. was able to prime mice for development of CTLs and to restimulate the spleen cells in vitro. PECs treated with UV-inactivated RSV are lysed as efficiently as those treated with infectious virus; in this respect RSV resembles viruses such as Sendai, with its powerful fusion protein (12), and differs, for example, from influenza virus: inactivated influenza virus is very poor at priming for CTLs (24) and does not form a target for them (1). Anti-RSV CTLs lysed RSV-treated PECs whether the PECs were treated with virus for 2 or 16 h, but the extent of lysis was more variable after treatment for 2 h.

We now wish to investigate the role of T cells in RSV infections in vivo, both in pathogenesis and protection against infection.

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