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Cytotoxic T-Lymphocyte Precursor Frequencies in BALB/c Mice after Acute Respiratory Syncytial Virus (RSV) Infection or Immunization with a Formalin-Inactivated RSV Vaccine

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A better understanding of the immune response to live and formalin-inactivated respiratory syncytial virus (RSV) is important for developing nonlive vaccines. In this study, major histocompatibility complex (MHC) class I- and II-restricted, RSV-specific cytotoxic T-lymphocyte precursor (CTLp) frequencies were determined in bronchoalveolar lavage (BAL) samples and spleen lymphocytes of BALB/c mice intranasally infected with live RSV or intramuscularly inoculated with formalin-inactivated RSV (FI-RSV). After RSV infection, both class I- and class II-restricted CTLps were detected by day 4 or 5 postinfection (p.i.). Peak CTLp frequencies were detected by day 7 p.i. The class II-restricted CTLp frequencies in the BAL following RSV infection were less than class I-restricted CTLp frequencies through day 14 p.i., during which class I-restricted CTLp frequencies remained elevated, but then declined by 48 days p.i. The frequencies of class II-restricted CTLps in the BAL were 2- to 10-fold less than those of class I-restricted CTLps. For spleen cells, frequencies of both MHC class I- and II-restricted CTLps to live RSV were similar. In contrast, class II-restricted CTLps predominated in FI-RSV-vaccinated mice. RSV challenge of vaccinated mice resulted in an increase in the frequency of class I-restricted CTLps at day 3 p.i. but did not enhance class II-restricted CTLp frequencies. These studies demonstrate differences in the CTLp response to live RSV infection compared with FI-RSV immunization and help define possible mechanisms of enhanced disease after FI-RSV immunization. In addition, these studies provide a quantitative means to address potential vaccine candidates by examining both MHC class I- and II-restricted CTLp frequencies.

Respiratory syncytial virus (RSV) infection in infants and young children often results in lower respiratory tract disease and is a high priority for vaccine development (1, 2). Attempts to develop an effective live, inactivated, or subunit vaccine have been unsuccessful (24, 25, 28). Early efforts at vaccinating young children with a formalin-inactivated RSV (FI-RSV) vaccine failed to protect the children from naturally acquired infection and actually enhanced lower respiratory tract disease upon later virus infection (2, 15, 24, 25). This enhanced disease has created concern about the safety of any nonlive RSV vaccine and, consequently, understanding the pathogenesis of FI-RSV-induced enhanced disease is critically important to vaccine development. Studies with BALB/c mice suggest that induction of memory T cells producing Th2-like cytokines, as a result of FI-RSV vaccination, may be key to the pathogenesis of enhanced disease (6, 16, 28, 32, 40). Th2-like cytokine mRNA has been demonstrated in cells from lung tissue or bronchoalveolar lavage (BAL) specimens after RSV challenge of FI-RSV-immunized mice (17, 32, 40). In addition, in vivo studies using antibody (Ab) blockade showed that the enhanced histopathology in FI-RSV-immune mice challenged with live virus could be eliminated by using anti-interleukin-4 (IL-4) and anti-IL-10 Abs but not anti-IL-12 Abs (6). Recent evidence suggests that $CD8^+$ T lymphocytes may be important in directing the type of inflammatory response to RSV in challenge of G glycoprotein-sensitized mice (21, 31).

One aspect of the FI-RSV immune response that has not been well characterized is the cytotoxic T-lymphocyte (CTL) response. There is limited information on major histocompatibility complex (MHC) class I-restricted CTLs after FI-RSV immunization (29), while the information about the CTL response after live-RSV infection has been well documented. Several studies have shown class I-restricted CTLs to kill predominantly target cells expressing the M, N, or F RSV protein (5, 7, 9, 26, 29, 41). The role of CTLs in the immune response to RSV is well illustrated by in vivo depletion studies with BALB/c mice (8, 18, 30). These studies suggest that both $CD4^+$ (class II) and $CD8^+$ lymphocytes are important for clearing RSV and that both contribute to the inflammatory response associated with infection. A vaccinia virus construct expressing RSV membrane-associated, nonglycosylated protein M2 has been affiliated with short-term protection in the BALB/c mouse (7). This protein does not induce neutralizing Abs, and therefore, protection likely is mediated by CTLs. Passive transfer of $CD8^+$ T lymphocytes has been associated with both clearance of the virus and enhanced histopathology (1).

In this report, we describe studies of CTL precursor (CTLp) frequencies in both live-RSV-infected and FI-RSV-immunized mice for MHC class I- and class II-restricted target cells. These studies demonstrate clear differences in the CTLp response between RSV and FI-RSV immunizations and provide additional approaches to identifying potential FI-RSV-induced enhanced disease mechanisms.

MATERIALS AND METHODS

Animals. Four- to 6-week-old, specific-pathogen-free female BALB/c mice were purchased from Harlan Sprague-Dawley Laboratories (Indianapolis, Ind.). The mice were housed in microisolator cages and fed sterilized water and food ad libitum.

Viruses and FI-RSV vaccine. The A2 (Long) strain of RSV and human parainfluenza virus type 3 (HPIV3) were grown in Vero cells in tissue culture medium

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(TCM) consisting of RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Salt Lake City, Utah), 1% L-glutamine, and 1% antibiotic-antimycotic (all from GIBCO). Upon the appearance of cytopathic effects, the medium was decanted and replaced with a minimal volume of Dulbecco's modified phosphate-buffered saline and frozen at −70°C. The flask was thawed, and any remaining adherent cells were scraped off with a cell scraper (Costar, Cambridge, Mass.) and collected. The cells and supernatant were centrifuged at 2,000 × *g* for 15 min at 4°C. The resulting supernatant was collected, divided into aliquots, and stored at −70°C. A portion of the virus stock was purified by using a discontinuous sucrose gradient as previously described (27). The titer was determined by methylcellulose plaque assay on Vero and HEp-2 cells.

FI-RSV and FI-HPIV3 vaccine was prepared as described previously (40). Briefly, 1 part formalin (Sigma, St. Louis, Mo.) was incubated with 4,000 parts clarified virus lysate for 3 days at 37°C and pelleted by centrifugation for 1 h at 50,000 × *g*. The volume of virus was adjusted to a 1:25 dilution of the original volume in minimum essential medium (MEM; GIBCO) and subsequently precipitated with aluminum hydroxide (4 mg/ml; Sigma), resuspended in 1/100 of the original volume in serum-free MEM, and stored at 4°C.

Infection, immunization, challenge, and sampling. Mice were anesthetized by intraperitoneal administration of 2,2,2-tribromoethanol (Avertin) and then infected intranasally (i.n.) with 10^6 PFU of RSV or HPIV3 or intramuscularly immunized with a 10^6 PFU equivalent of FI-RSV or FI-HPIV3 in the superficial gluteal muscle. A portion of the mice immunized with FI-RSV or FI-HPIV3 was i.n. challenged 3 weeks after immunization with 10^6 PFU of live RSV or HPIV3. Prior to removal of BAL or spleens on days 3, 4, 5, 7, 10, 14, and 48 postinfection (p.i.), mice were anesthetized by intraperitoneal administration of 2,2,2-tribromoethanol and exsanguinated by severing the right caudal artery. All organs were collected on ice in Hanks balanced salt solution.

MHC class I- and II-restricted CTLp assays. The class I-restricted target cells used were the mouse mastocytoma line P815 (ATCC TIB 64), and the class II-restricted target cells were a subclone of the B-cell lymphoma line A20 (ATCC TIB 208). Both cell lines were maintained in RPMI 1640 (GIBCO) containing 10% FBS (Hyclone) plus 1% antibiotic-antimycotic (GIBCO). The respective target cells were prepared by suspending 10^6 cells in 1.0 ml of serum-free MEM (GIBCO) containing 10^7-PFU/ml RSV (or a multiplicity of infection of 10 PFU/cell) in cell lysate (or a comparable dilution of uninfected cell control lysate) for 18 h at 37°C, followed by addition of 1.0 ml of MEM containing 10% FBS and 200 μCi of ^{51}Cr (Na_2CrO_4; Amersham, Arlington Heights, Ill.), and incubating them for an additional 2 h at 37°C. The cells were then washed and resuspended to an appropriate concentration in TCM comprised of suspension-MEM (GIBCO) containing 10% FBS (Hyclone), 1% essential amino acids, 2% nonessential amino acids, 2% sodium pyruvate, 2% L-glutamine, 1% antibiotic-antimycotic (all from GIBCO), and 50 μM 2-mercaptoethanol (Sigma).

Virus-specific CTLp prevalence was determined by using a modification of a well-established limiting-dilution assay (20, 30, 33, 35–37). In brief, different dilutions of effector cells in 0.1 ml of TCM were added to wells (24 wells/dilution) of round-bottom, 96-well microtiter plates (Costar) with 0.1 ml of antigen (Ag)-presenting cells (APCs). The APCs were syngeneic splenocytes that had been incubated in a serum-free MEM (GIBCO) containing 1,000-PFU/ml RSV for 2 to 3 h at 37°C and resuspended at 10^7 cells/ml in TCM containing 20% EL4.IL-2 supernatant (the lymphoma cell line EL4.IL-2 [ATCC TIB 181] endogenously secretes IL-2). The effector cells and APCs were incubated at 37°C for 7 days in a humidified atmosphere. The contents of individual wells were then divided in two, placed into replica plates, and incubated for 6 h with 10^4 ^{51}Cr-labeled, RSV-infected or mock-infected target cells. The virus-specific CTLp frequency was estimated by using linear regression and 95% confidence intervals about the slope of the regression line and plotting the number of cells versus the number of nonresponding cultures. A responding well was defined as one in which the mean ^{51}Cr release from RSV-infected targets plus effector cells was ≥3 standard deviations from the mean ^{51}Cr release from control wells containing uninfected target cells plus effector cells. The virus-specific CTLp frequency was estimated according to the Poisson equation at the 37% nonresponding culture point (F_0) along the slope of the linear regression line. The 95% confidence intervals were used to determine significance, which is indicated by $P < 0.05$.

Confirmation of MHC class II-specific cytolysis. To confirm class II-specific cytolysis, purified anti-I-A^d/I-E^d monoclonal Ab 2G9 (PharMingen) was incubated with effector cells and either P815 or A20 target cells. The purified anti-I-A^d/I-E^d monoclonal Ab blocked the cytolysis of the A20 target cells but not that of P815 target cells, demonstrating that cytolysis of A20 target cells was MHC class II restricted, as expected (data not shown).

RESULTS

Primary CTLp frequencies after live-RSV infection. Limiting-dilution analysis was performed during the acute immune response to i.n. RSV infection (Tables 1 and 2). Significant numbers of both MHC class I- and II-restricted, virus-specific CTLps could be detected in the BAL (Table 1) and spleen (Table 2) as early as days 4 (spleen) and 5 (BAL) p.i. The class I-restricted CTLp frequencies at day 5 p.i. in the BAL were significantly ($P < 0.05$) higher (Table 1; 1:46,500 to 1:50,200) than the class II-restricted CTLp frequencies (Table 1; 1:145,000 to 1:162,100). The frequencies of both class I-restricted CTLps remained relatively invariant through day 12 p.i. CTLp frequencies ranged from 1:5,300 at day 7 p.i. to 1:7,500 at day 14 p.i. The frequencies of class II-restricted CTLps were similarly stable, ranging from 1:22,800 at day 7 p.i. to 1:38,000 at day 14 p.i. However, the frequencies of both class I-and II-restricted CTLps examined at 48 days p.i. showed an approximately 10-fold decline (Table 1). In contrast, the class I-and II-restricted CTLp frequencies in the spleen were similar. The class I-restricted CTLp frequencies remained stable from day 5 to day 12 p.i. (Table 2; range, 1:2,200 at day 5 to 1:6,400 at day 12) and then decreased about sevenfold by day 48 p.i. (Table 2; range, 1:26,900 to 1:33,400). The class II-restricted CTLp frequency reached a maximum on day 10 p.i. (Table 2; range, 1:2,000 to 1:2,900) and decreased about eightfold by day 48 p.i. (Table 2; range, 1:14,200 to 1:21,200). Both the kinetics and frequencies of class I-restricted CTLps are comparable to those observed with other respiratory virus infections in mice, including Sendai and influenza virus infections (10–12). The RSV-specific, class II-restricted CTLp frequencies have not been previously reported but are similar to those reported for Sendai virus (20). Heterologous virus control infection with HPIV3 did not generate a detectable RSV-specific CTLp frequency (>10^6) in either the BAL (Table 1) or the spleen (Table 2).

Primary CTLp frequencies after FI-RSV vaccination. The CTLp frequencies in the spleen after FI-RSV vaccination were qualitatively different than those observed after live-RSV challenge noted above (i.e., higher frequencies of class II-restricted CTLps and lower frequencies of class I-restricted CTLps; Table 3). There were too few BAL cells after FI-RSV vaccination

TABLE 1. RSV-specific CTLp frequencies for MHC class I- and II-restricted lymphocytes isolated from BAL fluid of BALB/c mice at various time points post-i.n. infection with RSV or an HPIV3 control[a]

No. of days p.i.[b]	Virus	MHC class	CTLp frequency range[c]
3	RSV	I	>10^6
3	RSV	II	10^6
5	RSV	I	1:46,500–1:50,200
5	RSV	II	1:145,000–1:162,100
7	RSV	I	1:5,300–1:6,500
7	RSV	II	1:22,800–1:28,500
7	HPIV3	I	>10^6
10	RSV	I	1:2,800–1:6,300
10	RSV	II	1:25,500–1:32,200
10	HPIV3	I	>10^6
14	RSV	I	1:2,500–1:7,500
14	RSV	II	1:12,500–1:38,000
48	RSV	I	1:120,800–1:230,000
48	RSV	II	1:210,500–1:>10^6

[a] Three individual mice were examined at each time point for the frequencies of class I-and II-restricted CTLps, and the range of frequencies is shown.
[b] Number of days after RSV infection.
[c] The 95% confidence limits for all of the CTLp frequencies shown ranged from 1,200 to 26,300. The limiting-dilution protocol used ^{51}Cr-labeled, RSV-infected target cells and uninfected target cells to determine the background. The values for uninfected targets were always >1:500,000 and are not shown. The CTLp frequency for naive animals was >1:10^6.

TABLE 2. RSV-specific CTLp frequencies for MHC class I- and II-restricted lymphocytes isolated from the spleens of BALB/c mice at various time points post-i.n. infection with RSV or an HPIV3 control[a]

No. of days p.i.[b]	Virus	MHC class	CTLp frequency range[c]
3	RSV	I	$>10^6$
3	RSV	II	$>10^6$
4	RSV	I	1:29,200–1:37,900
4	RSV	II	1:71,800–1:75,700
5	RSV	I	1:2,200–1:8,300
5	RSV	II	1:2,300–1:12,100
7	RSV	I	1:4,800–1:6,000
7	RSV	II	1:5,500–1:6,300
7	HPIV3	I	$>10^6$
10	RSV	I	1:5,000–1:6,300
10	RSV	II	1:2,000–1:2,900
10	HPIV3	I	$>10^6$
12	RSV	I	1:4,000–1:6,400
12	RSV	II	1:2,200–1:3,300
48	RSV	I	1:26,900–1:33,400
48	RSV	II	1:14,200–1:21,200

[a] Three individual mice were examined at each time point for the frequencies of class I-and II-restricted CTLps, and the range of frequencies is shown.
[b] Number of days after RSV infection.
[c] The 95% confidence limits for all of the CTLp frequencies shown ranged from 710 to 1,800. The limiting-dilution protocol used ^{51}Cr-labeled, RSV-infected target cells and uninfected target cells to determine the background. The values for uninfected targets were always >1:500,000 and are not shown. The CTLp frequency for naive animals was $>1:10^6$.

to study CTLp frequencies. In the spleen, class I-restricted CTLp frequencies were the highest at day 10 (range, 1:14,600 to 1:18,700) and rapidly decreased to between 1:38,500 and 1:65,200 by day 48 p.i. (Table 3). Class II-restricted CTLp frequencies were higher than class I-restricted CTLp frequencies throughout the study period. The class II-restricted CTLps reached their highest numbers at day 10 p.i. (Table 3; range, 1:1,700 to 1:3,300) and decreased only about twofold at day 48 p.i. (Table 3; range, 1:2,700 to 1:3,900). RSV-specific CTLp frequencies could not be detected ($>10^6$) following immunization with FI-HPIV3 (Table 3).

CTLp frequencies after RSV challenge of FI-RSV-vaccinated mice. Upon live-RSV challenge of FI-RSV-vaccinated mice, a dramatic increase in class I-restricted CTLp frequencies was observed, as well as an initial decline in the class II-restricted CTLp frequency (Table 4). The class II-restricted CTLp frequency ranges decreased from 1:11,900 to 1:12,200 on day 0 to 1:19,700 to 1:24,600 on day 3 p.i. and then increased to between 1:8,200 and 1:12,700 on day 4 p.i. and to 1:3,100 to 1:8,200 on day 6 p.i. (Table 4). In contrast, the class I-restricted CTLp frequencies increased approximately 10-fold following challenge of FI-RSV-vaccinated mice, ranging between 1:111,300 and 1:113,600 at day 0 (3 weeks post-FI-RSV immunization) and between 1:12,600 and 1:14,400 at day 3 p.i. (Table 4). As expected, the CTLp frequencies detected were higher earlier in mice previously immunized (with FI-RSV) than in unimmunized mice (Table 4). HPIV3 infection, FI-HPIV3 immunization, or HPIV3 challenge of FI-HPIV3-immunized mice did not generate significant RSV-specific CTLps during the periods examined (Table 4). RSV challenge of FI-HPIV3-immunized mice resulted in CTLp frequencies comparable to those observed in mice receiving RSV alone (Table 4). HPIV3 challenge of HPIV3-immunized mice also did not generate detectable RSV-specific CTLps (Table 4).

DISCUSSION

In this study, we examined RSV-specific CTLp frequencies in BAL samples and spleen cells of BALB/c mice after live-virus infection, compared to FI-RSV immunization. After live-RSV infection, both MHC class I-and II-restricted CTLps were noted, with class I-restricted CTLps most common in BAL cells throughout the study, and in spleen cells early in the acute response phase. By day 10 p.i. in the spleen, class II-restricted CTLps were more frequent than class I CTLps. In contrast, FI-RSV immunization induced a much higher frequency of class II-restricted CTLps through day 14 p.i. This difference between the live-RSV and FI-RSV immunizations demonstrates the value of using CTLp frequencies to quantitate the CTL response and the importance of examining both class I-and II-restricted CTLp responses.

The preponderance of class II-restricted CTLps after FI-RSV vaccination was expected, given the noninfectious nature of FI-RSV. As an extracellular Ag, it must be endocytosed for processing, which is a route that favors class II Ag presentation but does not necessarily limit class I presentation. The delay observed in the development of the class I-restricted response in FI-RSV-immunized mice may reflect inefficient transport of this extracellular Ag to the endoplasmic reticulum, the site where class I molecules are loaded with peptides for presentation to $CD8^+$ T lymphocytes. The extracellular source of RSV Ag affiliated with FI-RSV immunization has also been

TABLE 3. RSV-specific CTLp frequencies for MHC class I- and II-restricted lymphocytes isolated from the spleens of BALB/c mice at various time points postvaccination with FI-RSV or an FI-HPIV3 control[a]

No. of days p.i.[b]	Ag	MHC class	CTLp frequency range[c]
3	FI-RSV	I	$>10^6$
3	FI-RSV	II	$>10^6$
4	FI-RSV	I	1:147,000–1:365,300
4	FI-RSV	II	1:21,600–1:43,400
5	FI-RSV	I	1:80,500–1:183,000
5	FI-RSV	II	1:8,800–1:10,900
7	FI-RSV	I	1:28,300–1:55,600
7	FI-RSV	II	1:2,000–1:3,800
7	FI-HPIV3	I	$>10^6$
10	FI-RSV	I	1:14,600–1:18,700
10	FI-RSV	II	1:1,700–1:3,300
10	FI-HPIV3	I	$>10^6$
12	FI-RSV	I	1:15,800–1:31,100
12	FI-RSV	II	1:1,500–1:2,600
48	FI-RSV	I	1:38,500–1:65,200
48	FI-RSV	II	1:2,700–1:3,900

[a] Three individual mice were examined at each time point for the frequencies of class I-and II-restricted CTLps, and the ranges are shown.
[b] Number of days after immunization with FI-RSV.
[c] The 95% confidence limits for class I-restricted CTLps ranged from 1,000 to 9,000. The 95% confidence limits for class II-restricted CTLPs ranged from 720 to 1,300. The limiting-dilution protocol used ^{51}Cr-labeled, RSV-infected target cells and uninfected target cells to determine the background. The values for uninfected targets were always >1:500,000 and are not shown.

TABLE 4. RSV-specific CTLp frequencies for MHC class I- and II-restricted lymphocytes isolated from the spleens of BALB/c mice challenged with RSV or HPIV3 3 weeks after intramuscular immunization with FI-RSV or FI-HPIV3[a]

No. of days p.i.[b]	Virus		MHC class	CTLp frequency range[c]
	Primary	Secondary		
0	FI-RSV		I	1:111,300–1:113,600
0	FI-RSV		II	1:11,900–1:12,200
3	FI-RSV	RSV	I	1:12,600–1:14,400
3	FI-RSV	RSV	II	1:19,700–1:24,600
4	FI-RSV	RSV	I	1:2,300–1:3,900
4	FI-RSV	RSV	II	1:8,200–1:12,700
6	FI-RSV	RSV	I	1:980–1:5,700
6	FI-RSV	RSV	II	1:3,100–1:8,200
3	RSV		I	$>10^6$
3	RSV		II	$>10^6$
6	RSV		I	1:4,800–1:6,000
6	RSV		II	1:5,800–1:6,500
0	FI-HPIV3		I	$>10^6$
3	FI-HPIV3	RSV	I	$>10^6$
5	FI-HPIV3	RSV	I	1:14,200–1:24,900[d]
3	HPIV3		I	$>10^6$
5	HPIV3		I	$>10^6$
3	FI-HPIV3	HPIV3	I	1:856,300–$>10^6$
5	FI-HPIV3	HPIV3	I	$>10^6$

[a] Three individual mice were examined at each time point for the frequencies of class I-and II-restricted CTLps, and the frequency ranges are shown.
[b] Number of days after RSV challenge.
[c] The 95% confidence limits for the CTLp frequencies shown ranged from 680 to 1,800. The limiting-dilution protocol used ^{51}Cr-labeled, RSV-infected target cells and uninfected target cells to determine the background. The values for uninfected targets were always >1:500,000 and are not shown. The RSV-specific CTLp frequencies for age-matched control mice infected with RSV alone are shown.
[d] The 95% confidence limits for the CTLp frequencies shown ranged from 500 to 720.

suggested as responsible for the induction of memory T cells that produce Th2-like rather than Th1-like cytokines (6, 32, 40). Class II-restricted CTL responses have been noted for a number of other RNA viruses, including parainfluenza and Sendai viruses (4, 10, 14), measles viruses (38, 39), and coronavirus (19) in the family *Paramyxoviridae* and recently for influenza virus (13, 34) in the family *Orthomyxoviridae*. Limiting dilution analysis of parainfluenza virus-specific CD4$^+$ and CD8$^+$ T lymphocytes from the peripheral blood of adults showed relatively high frequencies for both CD4$^+$ (1:4,700) and CD8$^+$ (1:2,500) CTLps (10). Measles virus infection in humans has been shown to induce both HLA class I-and class II-restricted CTLs specific to the transmembrane fusion glycoprotein (39). In mice, Sendai virus-specific class II-restricted T-cell responses have been investigated and defined (4, 13). Class II-restricted, Sendai virus-specific T-cell hybridomas have been generated, and their reactivity has been characterized to the level of specific peptide sequences in the matrix protein (4). Similarly, class II-restricted (CD4$^+$) precursors were detected in mice during the acute immune response to Sendai virus (13, 14). Mouse hepatitis virus, a coronavirus, has been found to induce only class II-restricted CTLs in both BALB/c and C57BL/6 mice (19). Thus, generation of class II-restricted cells probably is a common component of the immune response to RNA viruses.

Although this is the first study to examine MHC class I-and II-restricted, RSV-specific CTLp frequencies, a number of investigators have studied RSV-specific CTLs. Most of this work has focused on class I-restricted CTLs (1, 5, 7, 18, 26, 29), and one study identified class II-restricted CTLs in cell lines developed from spleen cells from BALB/c mice and peripheral blood mononuclear cells from humans (29). In this study, no effector CTLs were detected after FI-RSV immunization. The inability to detect RSV-specific effector CTLs after FI-RSV immunization may be due to differences in sensitivity and culture conditions between bulk-culture CTL assays and limiting-dilution culture assays.

It is appealing to speculate that the shift in CTLp frequency from a predominately class II-restricted response following FI-RSV vaccination to a predominately class I-restricted response following live-virus challenge is related to the shift from Th2-type cytokines toward Th1-type cytokines after RSV infection in FI-RSV-immunized mice (40). The data in Table 4 shows that the CTLp response generated by FI-RSV immunization, although predominantly class II restricted, is also significantly class I restricted. One possible mechanism for generating class I-restricted CTLps is by heat shock protein channeling of RSV peptides to the class I pathway, as has been reported for tumor peptides (3). Subsequent challenge of FI-RSV-immune mice with live RSV would then rapidly expand memory CD8$^+$ T lymphocytes and limit the Th2-type phenotype, as has been previously described (21, 31). This could offer a possible explanation for the lack of enhanced disease in FI-RSV-vaccinated children who experienced a second RSV infection. Further studies of the frequency and characteristics of RSV-specific CTLps should help to characterize disease-enhancing components of the immune response to RSV.

REFERENCES

1. **Cannon, M. J., P. J. Openshaw, and B. A. Askonas.** 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J. Exp. Med. **168:**1163–1168.
2. **Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette.** 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. **89:**449–463.
3. **Ciupitu, A. M., M. Petersson, C. L. O'Donnell, K. Williams, S. Jindal, R. Kiessling, and R. M. Welsh.** 1998. Immunization with a lymphocytic choriomeningitis virus peptide mixed with heat shock protein 70 results in protective antiviral immunity and specific cytotoxic T lymphocytes. J. Exp. Med. **187(5):**685–691.
4. **Cole, G. A., Y. Tao, T. L. Hogg, K. W. Ryan, and D. L. Woodland.** 1995. Binding motifs predict major histocompatibility complex class II-restricted epitopes in the Sendai virus M protein. J. Virol. **69:**8057–8060.
5. **Connors, M., P. L. Collins, C.-Y. Firestone, and B. R. Murphy.** 1991. Respiratory syncytial virus (RSV) F, G, M2 (22K), and N proteins each induce resistance to RSV challenge, but resistance induced by M2 and N proteins is relatively short-lived. J. Virol. **65:**1634–1637.
6. **Connors, M., N. A. Giese, A. B. Kulkarni, C.-Y. Firestone, H. C. Morse III, and B. R. Murphy.** 1994. Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. J. Virol. **68:**5321–5325.
7. **Connors, M., A. B. Kulkarni, P. L. Collins, C.-Y. Firestone, K. L. Holmes, H. C. Morse III, and B. R. Murphy.** 1992. Resistance to respiratory syncytial virus (RSV) challenge induced by infection with a vaccinia virus recombinant expressing the RSV M2 protein (Vac-M2) is mediated by CD8$^+$ T cells, while that induced by Vac-F or Vac-G recombinants is mediated by antibodies. J. Virol. **66:**1277–1281.
8. **Connors, M., A. B. Kulkarni, C.-Y. Firestone, K. L. Holmes, H. C. Morse III, A. V. Sotnikov, and B. R. Murphy.** 1992. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-

immunized BALB/c mice is abrogated by depletion of CD4$^+$ T cells. J. Virol. **66:**7444–7451.
9. **Corvaisier, C., G. Guillemin, C. Bourgeois, J. B. Bour, E. Kohli, and P. Pothier.** 1993. Identification of T-cell epitopes adjacent to neutralizing antigenic domains on the fusion protein of respiratory syncytial virus. Res. Virol. **144:**141–150.
10. **Dhib-Jalbut, S., and S. Jacobson.** 1994. Cytotoxic T cells in paramyxovirus infection in humans. Curr. Top. Microbiol. Immunol. **189:**109–121.
11. **Doherty, P. C.** 1996. Cytotoxic T cell effector and memory function in viral immunity. Curr. Top. Microbiol. Immunol. **206:**1–14.
12. **Doherty, P. C., S. Hou, and R. A. Tripp.** 1994. CD8$^+$ T-cell memory to viruses. Curr. Opin. Immunol. **6:**545–552.
13. **Doherty, P. C., D. J. Topham, and R. A. Tripp.** 1996. Establishment and persistence of virus-specific CD4$^+$ and CD8$^+$ T cell memory. Immunol. Rev. **150:**23–44.
14. **Ewing, C., D. J. Topham, and P. C. Doherty.** 1995. Prevalence and activation phenotype of Sendai virus-specific CD4$^+$ T cells. Virology **210:**179–185.
15. **Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn.** 1969. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am. J. Epidemiol. **89:**435–448.
16. **Graham, B. S.** 1996. Immunological determinants of disease caused by respiratory syncytial virus [see comments]. Trends Microbiol. **4:**290–293.
17. **Graham, B. S., G. S. Henderson, Y. W. Tang, X. Lu, K. M. Neuzil, and D. G. Colley.** 1993. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. J. Immunol. **151:**2032–2040.
18. **Graham, B. S., L. A. Bunton, P. F. Wright, and D. T. Karzon.** 1991. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J. Clin. Investig. **88:**1026–1033.
19. **Heemskerk, M. H., H. M. Schoemaker, W. J. Spaan, and C. J. Boog.** 1995. Predominance of MHC class II-restricted CD4$^+$ cytotoxic T cells against mouse hepatitis virus A59. Immunology **84:**521–527.
20. **Hou, S., and P. C. Doherty.** 1993. Partitioning of responder CD8$^+$ T cells in lymph node and lung of mice with Sendai virus pneumonia by LECAM-1 and CD45RB phenotype. J. Immunol. **150:**5494–5500.
21. **Hussell, T., C. J. Baldwin, A. O'Garra, and P. J. Openshaw.** 1997. CD8$^+$ T cells control Th2-driven pathology during pulmonary respiratory syncytial virus infection. Eur. J. Immunol. **27**(12)**:**3341–3349.
22. **Institute of Medicine.** 1985. New vaccine development: establishing priorities, diseases of importance in the United States. National Academy Press, Washington, D.C.
23. **Institute of Medicine.** 1986. New vaccine development: establishing priorities, diseases of importance in developing countries. National Academy Press, Washington, D.C.
24. **Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart.** 1969. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am. J. Epidemiol. **89:**405–421.
25. **Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. Jensen, and R. H. Parrott.** 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. **89:**422–434.
26. **Kulkarni, A. B., M. Connors, C.-Y. Firestone, H. C. Morse III, and B. R. Murphy.** 1993. The cytolytic activity of pulmonary CD8$^+$ lymphocytes, induced by infection with a vaccinia virus recombinant expressing the M2 protein of respiratory syncytial virus (RSV), correlates with resistance to RSV infection in mice. J. Virol. **67:**1044–1049.
27. **Mbiguino, A., and J. Menezes.** 1991. Purification of human respiratory syncytial virus: superiority of sucrose gradient over percoll, renografin, and metrizamide gradients. J. Virol. Methods **31:**161–170.
28. **Murphy, B. R., S. L. Hall, A. B. Kulkarni, J. E. Crowe, Jr., P. L. Collins, M. Connors, R. A. Karron, and R. M. Chanock.** 1994. An update on approaches to the development of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccines. Virus Res. **32:**13–36.
29. **Nicholas, J. A., K. L. Rubino, M. E. Levely, E. G. Adams, and P. L. Collins.** 1990. Cytolytic T-lymphocyte responses to respiratory syncytial virus: effector cell phenotype and target proteins. J. Virol. **64:**4232–4241.
30. **Openshaw. P. J.** 1995. Immunity and immunopathology to respiratory syncytial virus. The mouse model. Am. J. Respir. Crit. Care Med. **152:**S59–S62.
31. **Srikiatkhachorn, A., and T. J. Braciale.** 1997. Virus-specific CD8$^+$ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection. J. Exp. Med. **186**(3)**:**421–432.
32. **Tang, Y. W., and B. S. Graham.** 1995. Interleukin-12 treatment during immunization elicits a T helper cell type 1-like immune response in mice challenged with respiratory syncytial virus and improves vaccine immunogenicity. J. Infect. Dis. **172:**734–738.
33. **Taswell, C.** 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J. Immunol. **126:**1614–1619.
34. **Taylor, S. F., and B. S. Bender.** 1995. Beta 2-microglobulin-deficient mice demonstrates class II MHC restricted anti-viral CD4$^+$ but not CD8$^+$ CTL against influenza-sensitized autologous splenocytes. Immunol. Lett. **46:**67–73.
35. **Tripp, R. A., S. Hou, and P. C. Doherty.** 1995. Temporal loss of the activated L-selectin-low phenotype for virus-specific CD8$^+$ memory T cells. J. Immunol. **154:**5870–5875.
36. **Tripp, R. A., S. Hou, S. McMickle, J. Houston, and P. C. Doherty.** 1995. Recruitment and proliferation of CD8$^+$ T cells in respiratory virus infections. J. Immunol. **154:**6013–6021.
37. **Tripp, R. A., S. R. Sarawar, and P. C. Doherty.** 1995. Characteristics of the influenza virus-specific CD8$^+$ T cell response in mice homozygous for disruption of the H-21Ab gene. J. Immunol. **155:**2955–2959.
38. **UytdeHaag, F. G., R. S. van Binnendijk, M. J. Kenter, and A. D. Osterhaus.** 1994. Cytotoxic T lymphocyte responses against measles virus. Curr. Top. Microbiol. Immunol. **189:**151–167.
39. **van Binnendijk, R. S., J. P. Versteeg-van Oosten, M. C. Poelen, H. F. Brugghe, P. Hoogerhout, A. D. Osterhaus, and F. G. Uytdehaag.** 1993. Human HLA class I-and HLA class II-restricted cloned cytotoxic T lymphocytes identify a cluster of epitopes on the measles virus fusion protein. J. Virol. **67:**2276–2284.
40. **Waris, M. E., C. Tsou, D. D. Erdman, S. R. Zaki, and L. J. Anderson.** 1996. Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J. Virol. **70:**2852–2860.
41. **Welliver, R. C., D. A. Tristram, K. Batt, M. Sun, D. Hogerman, and S. Hildreth.** 1994. Respiratory syncytial virus-specific cell-mediated immune responses after vaccination with a purified fusion protein subunit vaccine. J. Infect. Dis. **170:**425–428.