Respiratory syncytial virus (RSV) is a common cause of upper respiratory tract infections but may—especially in young infants, the elderly, or immunocompromised individuals—cause severe lower respiratory tract infections (12). In rodent models, CD4+, as well as CD8+, RSV-specific T lymphocytes proved to be involved in both recovery from and immune pathogenesis of the infection (1, 2, 8, 19). Therefore, a fine balance must exist in these models between protective and disease-enhancing effects of virus-specific T lymphocytes. RSV-specific CD8+ cytotoxic T lymphocytes (CTL) against virtually all RSV proteins have been demonstrated to circulate in humans after RSV infection (4, 7, 9). CTL may be expected to play a crucial role in the clearance of RSV infections, but their role in protection and immune pathology remains unclear. Therefore, the identification of CTL epitopes of human RSV may contribute to future studies concerning the role of CTL in pathogenesis and protection from RSV infection. Here we describe the functional characterization of CD8+ cytotoxic T-cell clones (TCC) generated from two infants who had just recovered from severe RSV infection. These TCC were also used to identify, for the first time, HLA class I-restricted CTL epitopes of RSV.

Peripheral blood mononuclear cells (PBMC) were collected from two infants 4 weeks after a severe, laboratory-confirmed RSV infection. At the time of infection, they were 1 and 2 months old and had both been admitted to the intensive care unit. B-lymphoblastic cell lines (B-LCL) were generated by Epstein-Barr virus transformation (22), infected with RSV A2 (ATCC VR1322), and UV irradiated (24) to serve as autologous antigen-presenting cells (APC). PBMC (3 × 10^6/well) in 96-well round-bottom plates were pulsed with APC (10^5/well) and expanded in RPMI 1640 medium supplemented with antibiotics, 10% pooled and heat-inactivated human serum, and recombinant human interleukin-2 (IL-2; 50 IU/ml). After 2 weeks, T cells were harvested and cloned by limiting dilution using phytohemagglutinin stimulation as previously described (25). TCC thus generated were expanded and tested for RSV specificity by [3H]thymidine incorporation assays as previously described (22). All of the RSV-specific TCC proved to be of the CD8+ phenotype in fluorescence-activated cell sorter analysis. TCC whose specificity for RSV was confirmed by a second proliferation assay were tested for protein specificity by a gamma interferon (IFN-γ) ELIspot assay (Mabtech AB, Stockholm, Sweden). In this test, paraformaldehyde-fixed autologous B-LCL that had been infected with recombinant vaccinia viruses (rVV) expressing different RSV proteins (F, G, N, P, M2, SH, M, 1B, or 1C) were used as APC. Briefly, TCC (5 × 10^3/well) were incubated with APC (10^5/well) for 4 h, transferred to anti-human IFN-γ-coated plates, and incubated for another 18 h. The ELIspot assay was further performed in accordance with the kit manufacturer’s instructions. The results are shown as numbers of IFN-γ-producing cells (spots) per well.

Thirty-four RSV-specific TCC were generated from the PBMC of patient 1, as detected in a [3H]thymidine incorporation assay. Of these, 27 proved to be RSV F specific and 7 were RSV 1B specific in an IFN-γ ELIspot assay (Fig. 1A). Twenty-four RSV-specific TCC were generated from the PBMC of patient 2 as detected by a [3H]thymidine incorporation assay. Of these, 10 were RSV F specific and 14 proved to be RSV 1C specific in an IFN-γ ELIspot assay (Fig. 1B). None of the clones detected by the TCC were found to be negative by the IFN-γ ELIspot assay. Since the F protein is considered to be a major CTL target (4, 7, 9), further efforts were focused on the identification of CTL epitopes in the F protein. To this end, 18-mer peptide amides overlapping by 12 amino acids, together spanning the entire F protein (rVV expressing different RSV proteins (F, G, N, P, M2, SH, M, 1B, or 1C) were used as APC. Briefly, TCC (5 × 10^3/well) were incubated with APC (10^5/well) for 4 h, transferred to anti-human IFN-γ-coated plates, and incubated for another 18 h. The ELIspot assay was further performed in accordance with the kit manufacturer’s instructions. The results are shown as numbers of IFN-γ-producing cells (spots) per well.

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FIG. 1. Responses of RSV-specific TCC from patients 1 (A) and 2 (B) measured by an IFN-γ ELIspot assay using autologous B-LCL infected with RSV A2 or rVV expressing the RSV F, 1B, or 1C protein. Results are indicated as numbers of spots per well. No responses against the B-LCL infected with the other rVV were found (data not shown).
The HLA restriction of the recognition by the RSV F TCC was also determined by the IFN-γ ELISPOT assay using a set of allogeneic B-LCL with partially matched HLA types loaded with the respective minimal peptides, as shown in Fig. 3. Recognition of the RSV F epitope of patient 1 proved to be HLA B57 restricted. Recognition of the epitope of patient 2 proved to be C12 restricted.

Well-growing TCC of both specificities from each of the patients was also determined by the IFN-γ ELISPOT assay using APC pulsed with two additional sets of overlapping 8- to 12-mer peptides.

FIG. 2. Fine mapping of T-cell epitopes on the RSV F protein. Responsiveness of TCC from patients 1 (A) and 2 (B) to partially overlapping 18-mer peptide amides (n = 94) spanning the F protein was measured by an IFN-γ ELISPOT assay. Minimal peptide activation of TCC from patients 1 (C) and 2 (D) was measured in an IFN-γ ELISPOT assay using APC pulsed with two additional sets of overlapping 8- to 12-mer peptides.

FIG. 3. Determination of HLA restriction of RSV F protein recognition by specific TCC using a set of autologous and partially HLA class I-matched B-LCL for patients 1 (A) and 2 (B) by an IFN-γ ELISPOT assay.
patients were arbitrarily selected and tested for cytotoxic activity by a chromium release assay as previously described (23). Briefly, autologous 51Cr-labeled, RSV-infected B-LCL and control B-LCL or, in the case of the F-specific TCC of patient 2, minimal-peptide-loaded B-LCL and control B-LCL were incubated with TCC for 4 h at 37°C at an effector-to-target cell ratio of 10:1, which was found to be the most discriminative ratio in preliminary experiments. Spontaneous 51Cr release (target cells plus medium) and maximum 51Cr release (target cells plus Triton X-100) were determined in 12 identical wells. Supernatants were harvested and analyzed in a gamma counter. Assay results were accepted only when the spontaneous-to-maximum release ratio was <25%. All of the TCC tested showed RSV-specific lysis (Fig. 4A). Virus-specific production of cytokines in cell-free culture supernatant was measured as previously described (6). Cytotoxic responses were measured using commercially available sandwich enzyme-linked immunosorbent assay systems in accordance with the manufacturers’ instructions: IL-4 (CLB, Amsterdam, The Netherlands; detection limit, 7 pg/ml), IFN-γ (Medgenix Diagnostics, Fleurs, Belgium; detection limit, 25 pg/ml), IL-10 (Pharmingen, San Diego, Calif.; detection limit, 20 pg/ml). All of the TCC produced predominantly IFN-γ, indicating a type 1 phenotype in vitro (Fig. 4B).

In the present study, we identified two nine-mer CTL epitopes on the RSV fusion protein. To our knowledge, these are the first HLA class I-restricted CTL epitopes described for RSV in humans. We found CTL epitopes with an HLA restriction with a low prevalence in the population. But using the techniques described here on samples of more children with a recent RSV infection, CTL epitopes with more prevalent HLA restrictions may be identified.

The TCC found in the children studied were all CD8+ CTL with a type 1-like cytokine profile. No RSV-specific CD4+ T cells were detected in the samples of these infants, while the use of similar stimulation protocols did result in the identification of such cells in other systems (14, 17, 20, 22). Virus-specific CTL responses play a major role in the clearance of RSV infections but may also be involved in pathogenesis (1, 3, 5, 8, 15). In mice, enhanced lung pathology induced by priming with formalin-inactivated RSV has been associated with the absence of a CTL response (13, 21). In young children, a CTL response against RSV has been described but poor responses were found in younger and more severely ill patients (10, 16). The poor CTL response in young children has been suggested to be one of the possible causes of more severe disease. In these two patients, it was possible to detect RSV-specific cytotoxic TCC, showing that also in very young children with clinically severe infections, priming of a CTL response does occur, although we cannot say anything about the quantitative responses.

In conclusion, use of the IFN-γ ELISpot assay to determine epitope specificity proved to be sensitive and convenient, since only small numbers of T cells and APC were needed. This and similar studies may be important for future studies concerning...
the role of CTL in the pathogenesis of RSV infection in children.

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