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The 22,000-Kilodalton Protein of Respiratory Syncytial Virus Is a Major Target for K^d-Restricted Cytotoxic T Lymphocytes from Mice Primed by Infection

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Recombinant vaccinia viruses containing the 22-kilodalton protein (matrixlike or 22K protein) or phosphoprotein gene from respiratory syncytial virus were constructed. These recombinant viruses expressed proteins which were immunoprecipitated by appropriate respiratory syncytial virus antibodies and comigrated with authentic proteins produced by respiratory syncytial virus infection. The new recombinant viruses (and others previously described containing the attachment glycoprotein, fusion, or nucleoprotein genes of respiratory syncytial virus) were used to infect target cells for cultured polyclonal cytotoxic T lymphocytes generated from the spleens of BALB/c or DBA/2 mice primed by intranasal infection with respiratory syncytial virus. Respiratory syncytial virus-specific cytotoxic T lymphocytes (CTL) showed strong K^d (but not D^d)-restricted recognition of the 22K protein. As previously reported, the fusion protein and nucleoprotein were both seen by CTL, but recognition of these proteins was comparatively weak. There was no detectable recognition of other respiratory syncytial virus proteins tested (including phosphoprotein). 22K protein-specific splenic memory CTL persisted for at least 11 months after infection of BALB/c mice. Priming BALB/c mice with recombinant vaccinia virus containing the 22K protein gene induced respiratory syncytial virus-specific memory CTL at lower levels than that previously reported following infection with a similar recombinant containing the fusion protein gene. These data identify the 22K protein as a major target antigen for respiratory syncytial virus-specific CTL from $H\text{-}2^d$ mice primed by respiratory syncytial virus infection.

Cytotoxic T-lymphocytes (CTL) have an important role in clearing respiratory viruses from infected hosts but can either reduce (27, 29) or increase (11) the severity of lung pathologic changes. The viral proteins and epitopes recognized by CTL (in association with class I major histocompatibility complex [MHC] molecules) often differ from those which are dominant antigens for helper T cells (in association with class II MHC molecules) or for antibody molecules induced by virus infection or vaccination. In virus infections analyzed so far, human or murine CTL recognition sites frequently lie within relatively invariant internal viral proteins (20, 23, 31, 37, 47, 52) rather than within the more variable surface glycoproteins. Once an infection is established, antibody alone appears insufficient to eliminate virus from within cells. An understanding of CTL recognition patterns in different virus infections is required for rational vaccine development.

Human respiratory syncytial (RS) virus is a major cause of bronchiolitis and pneumonia in children. No safe, effective vaccine is available. Mice infected intranasally with human isolates of RS virus show replication of virus in the lungs (36, 45) from which virus-specific CTL can be isolated (44). CTL can be generated from the spleens of mice primed by infection of the respiratory tract with RS virus (2) and show cross-reactive recognition of natural isolates of RS virus (1). Human RS virus has 10 genes which encode 10 viral proteins. In previous work, we constructed recombinant vaccinia viruses (VV) which expressed five of these individual proteins and used these recombinants to examine the role of defined proteins in humoral and cell-mediated immunity to RS virus. Initial studies showed that vaccination of experimental animals with recombinant VV encoding attachment protein G or fusion protein F led to a reduction of the virus titer in the lungs on subsequent intranasal challenge with RS virus (42, 50), although it sometimes increased subsequent lung pathologic changes (43). The RS viral proteins recognized by CTL were also analyzed by use of these recombinant VV. CTL from human and murine donors primed by intranasal infection show some recognition of RS virus nucleoprotein (3), although lysis of targets expressing nucleoprotein is poor in comparison with that of targets infected with RS virus. Some CTL lines and clones obtained from human or murine donors primed by RS virus infection show recognition of F and of diverse serotypes of RS virus (10), whereas priming mice by infection with F protein VV induces RS virus serotype-specific memory CTL (34). Using the previously available panel of recombinant VV, the protein(s) recognized by most of the RS virus-specific CTL lines and clones generated from BALB/c mice primed by infection of the respiratory tract with RS virus were not identified (10; unpublished data). Lysis of RS virus-infected target cells by polyclonal CTL has been far stronger than that of cells infected with any one of the available recombinant VV. Therefore, it has been postulated that the major CTL recognition protein(s) of RS virus awaits identification (11, 34).

In the work reported here, recombinant VV that express two additional human RS virus proteins, the 22-kilodalton protein (envelope-associated and matrixlike protein [22K protein]) and phosphoprotein, were constructed and their expression was analyzed. These new recombinant viruses were used to infect target cells for CTL from BALB/c and

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DBA/2 mice primed by intranasal infection with RS virus. The 22K protein was strongly recognized by CTL from either mouse strain; targets expressing F or N protein were recognized weakly in comparison. In BALB/c mice, phosphoprotein (P) and (as previously reported) glycoprotein G were recognized little or not at all. Recognition of the 22K protein was restricted by K^d (not D^d) MHC class I molecules. In contrast to the strong RS virus-specific CTL responses reported after recombinant VV-F infection (34), priming mice by infection with recombinant VV-22K gave rise to spleen cell cultures with weak but detectable RS virus-specific CTL activity.

MATERIALS AND METHODS

Viruses and cells. RS virus (A2 strain) was obtained from E. J. Stott (Institute for Research on Animal Diseases, Compton, Berkshire, United Kingdom) and grown in HEp-2 cells (2). Infectivity was measured by the single-cell assay developed by Cannon (9). VV (WR strain) was grown in HEp-2 cells, and infectivity was measured by plaque assay in 24-well Costar plates. Recombinant VV containing the gene for RS virus fusion protein (VF 317, henceforth referred to as VV-F), attachment protein (VAG 301, henceforth referred to as VV-G), or nucleoprotein (VN 333, henceforth referred to as VV-N) have already been described (references 25, 42, and 50, respectively).

Construction of plasmids and isolation of recombinant viruses. A cDNA clone (K44) containing all but 21 nucleotides from the 5′ end of the 22K protein gene was isolated from an RS virus (A2 strain) cDNA library in phagemid vector pIBI76. A 0.9-kilobase *Bam*HI fragment coding for all but the first four amino-terminal amino acids of the 22K protein was ligated into the *Sma*I site of VV vector plasmid pAB174, which contains a VV early promoter derived from the *Hin*dIII F fragment of the VV genome (42). This promoter fragment contains an adjacent ATG codon that serves as the translational start codon for genes inserted in frame into a downstream *Bam*HI-*Sma*I polylinker. The resulting 22K-VV vector plasmids in the positive orientation with respect to the VV F (early) promoter would code for a chimeric protein that contains two additional amino acids at the amino terminus of the 22K protein and differs in amino acid sequence for four additional residues. The amino-terminal 22K protein sequence synthesized by the recombinant virus would be MGSPIPN, in comparison with the predicted 22K protein sequence, MSRRN, from the published RS virus 22K protein gene sequence (13). The differences in amino acid sequence result from lack of the first 21 nucleotides in cDNA clone K44 and addition of the promoter fragment ATG and the *Bam*HI-*Sma*I polylinker sequences in the 22K-VV vector plasmids.

Recombinant VV vector plasmids containing the *P* gene in either the positive or the negative orientation with respect to the VV 7.5K (early or late) promoter were constructed by inserting a cDNA fragment containing the entire coding region of the *P* gene into the *Sma*I site of VV vector plasmid pVV192.76, which contains the VV 7.5K promoter. Vector plasmid pVV192.76 was derived by ligation of the VV recombination cassette from VV vector pAB192, in which the VV 7.5K promoter is transcriptionally opposed to the VV thymidine kinase promoter (L. A. Ball, unpublished data), into the multiple cloning site of phagemid vector pIBI76 (International Biotechnologies, Inc.). Recombinant plasmids containing *P* gene cDNA inserts were screened for orientation with respect to the VV 7.5K promoter by nucleotide sequencing (21). The *P* gene cDNA insert was obtained by ligation of a restriction fragment coding for the amino-terminal 21 amino acids from an RS virus (strain 8/60) cDNA clone and a restriction fragment coding for the carboxyl-terminal 219 amino acids from an RS virus (strain A2) cDNA clone (P147). The P protein expressed in recombinant VV-P55-infected cells differs in sequence from the published P amino acid sequence of RS virus strain A2 by a single conservative change of lysine instead of arginine at amino acid residue 16 (41).

To isolate recombinant viruses, vector DNA and wild-type VV DNA were cotransfected into HEp-2 cells infected with temperature-sensitive mutant VV *ts*17 (14, 17, 24). Cotransfected or infected cells were incubated at the permissive temperature (33°C) for 6 h and then shifted to the restrictive temperature (39.5°C) to select for recombinant VV replication. Recombinant VV isolates were identified by dot blot hybridization (13) and subjected to three rounds of plaque purification before preparation of virus stocks (42). Recombinants VV-22K1 and VV-22K5 contained the 22K protein gene in the positive orientation with respect to the VV F promoter. Recombinant VV-P33 and recombinant VV-P55 contained the *P* gene in the negative and positive orientations, respectively, downstream of the VV 7.5K promoter.

Priming of mice by infection. For RS virus infection, female BALB/c or DBA/2 mice were lightly anesthetized with ether and 2×10^5 PFU of RS virus in 50 μl was instilled intranasally. For infection with VV, mice were shaved over the rump and tail base. After 2 to 24 h, 10 μl of VV stock (diluted to contain 10^6 to 5×10^6 PFU) was placed on the shaved area and distributed over about 1 cm^2 by 60 to 100 light scratches with a fine needle point. Mice were checked for formation of typical infective lesions on days 3 to 5.

In vitro culture of spleen cells and cytotoxicity assay. A standard ^{51}Cr assay (2) was used to assess cytotoxicity against cells prepared by virus infection. In brief, 1.5×10^7 splenic cells from primed mice (responders) were placed in upright 25-cm^2 tissue culture flasks. Spleen cells (3.75×10^6) were pelleted and incubated with 1 PFU of RS virus stock per cell for 2 h at 37°C to form stimulator cells. The stimulators were added (without washing) to the responders and made up to 15 ml with RPMI 10–2-mercaptoethanol. The flasks were incubated at 37°C in 5% CO_2 for 5 days. Target cells were infected with 2 PFU of RS virus per cell or 10 PFU of VV per cell for 2 h in a minimal volume before suspension at 10^6/ml for overnight incubation. Targets were labeled with ^{51}Cr before use. After incubation with CTL for 3 to 4 h at 37°C, supernatants were harvested for ^{51}Cr measurement.

RESULTS

Expression of 22K or P protein in recombinant VV-infected cells. Expression of the human RS virus 22K and P proteins in cells infected with recombinant VV containing genes for these proteins was assayed by immunoprecipitation with RS virus-specific antibodies (Fig. 1). Recombinants VV-22K1 and VV-22K5, which each contained the 22K protein gene in the positive orientation with respect to the VV F promoter, synthesised a 22K protein that comigrated with an RS virus-specific protein from infected cells (Fig. 1A). An additional protein species of approximately 25.5 kilodaltons was identified in an RS virus-infected cell lysate migrating just above the F2 protein (Fig. 1A, arrow), which was also identified by immunoprecipitation with monoclonal antibody 5H5 alone (data not shown) and by Routledge et al. (39) on

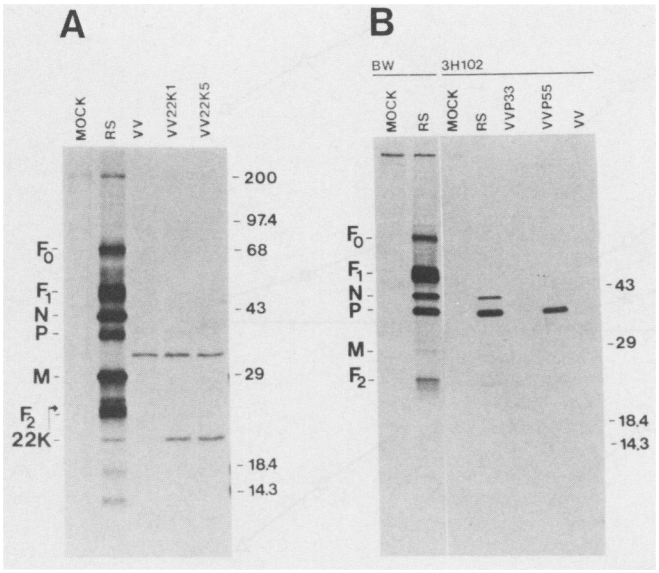

FIG. 1. Expression of RS virus proteins by recombinant VV. HEp-2 cells were infected with RS virus at a multiplicity of 1 to 3 infectious units per cell or with the VV at a multiplicity of 20. Mock- and virus-infected cells were incubated in methionine-free minimal essential medium for 30 min before being labeled with [^{35}S]methionine (50 μCi/ml) for 2 h. Mock- and RS virus-infected cells were labeled from 21 to 23 h postinfection, and VV-infected cells were labeled from 3.5 h postinfection. Infected-cell lysates were immunoprecipitated with various RS-specific antibodies, and the resulting samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 15% polyacrylamide gel. The samples shown in panel A are (left to right) from mock-infected or RS virus-infected cell lysates immunoprecipitated with polyclonal antiserum (bovine anti-RS virus serum from Burroughs Wellcome) and wild-type VV-infected (VV) and recombinant VV-infected (VV-22K1 and VV-22K5) cells immunoprecipitated with monoclonal antibody 5H5 (specific to the 22K protein). The arrow to the left of the autoradiogram shows the position of a second protein of approximately 25 kilodaltons (located just above the F2 band) which was immunoprecipitated from RS virus-infected cells in addition to the 22K protein with 22K protein-specific monoclonal antibody 5H5 (as previously observed by Routledge et al. [39] by Western blotting; data not shown). For panel B, cell lysates from control or recombinant VV-infected cells were immunoprecipitated with Burroughs Wellcome or phosphoprotein-specific monoclonal antibody 3H102. Phosphoprotein from RS virus-infected cells appeared to coprecipitate with nucleoprotein (see the text). The numbers to the right indicate molecular sizes in kilodaltons.

Western blots. Recombinant VV-22K5 was used in subsequent experiments unless otherwise specified.

In cells infected with recombinant VV-P55 containing the *P* gene in the positive orientation with respect to the VV 7.5K promoter, a protein that comigrated with the P protein of RS virus-infected cells was synthesised and identified by immunoprecipitation with monoclonal antibody 3H102 (Fig. 1B). No *P*-specific proteins were observed in cells infected with recombinant VV-P33, which contained the *P* gene in the negative orientation with respect to the 7.5K promoter. In RS virus-infected cells, both N and P proteins were immunoprecipitated with the P-specific monoclonal antibody. This may reflect a specific interaction between nucleocapsid and phosphoproteins of RS virus, as has been reported for other negative-strand viruses, such as vesicular stomatitis virus (6).

The 22K protein of RS virus is a major target antigen for CTL. To test CTL recognition of target cells infected with recombinant VV, CTL were generated from mice primed by RS virus infection by in vitro stimulation of spleen cells with RS virus. Target cells infected with recombinant VV-22K were lysed by CTL similarly to or to a greater extent than RS virus-infected targets (Table 1; Fig. 2). Recognition of recombinant VV-F-infected cells was weak by comparison, and no specific recognition of recombinant VV-P55- or VV-G-infected targets was seen (Fig. 2). Targets infected with recombinant VV-22K 1 or 5 were lysed equally (data not shown).

TABLE 1. RS virus-specific CTL from DBA/2 mice recognize 22K protein[a]

K/T ratio	% Specific ^{51}Cr release by targets infected with:					
	No virus	RS virus	VV-22K	VV-F	VV-N	Wild-type VV
3	2	9	29	7	4	4
7	5	19	49	10	8	7
14	11	29	63	19	16	11
28	17	41	76	32	24	17

[a] CTL from DBA/2 mice were tested against P815 cells uninfected or infected with various viruses. Similar CTL from BALB/c mice tested against these same targets gave almost identical results (data not shown). CTL from either mouse strain were tested simultaneously against L929 cells transfected with D^d and infected with various viruses, including RS virus and recombinant VV-22K, and induced no significant lysis (data not shown).

CTL from BALB/c mice primed 11 weeks (Fig. 3, upper panels) or 11 months (lower panels) before in vitro restimulation also lysed targets infected with recombinant VV-22K as strongly as targets infected with RS virus; recombinant VV-F and VV-N targets were lysed to a lesser degree, and VV-G targets were lysed similarly to control uninfected targets. L cells transfected with the K^d gene (L-K^d) and

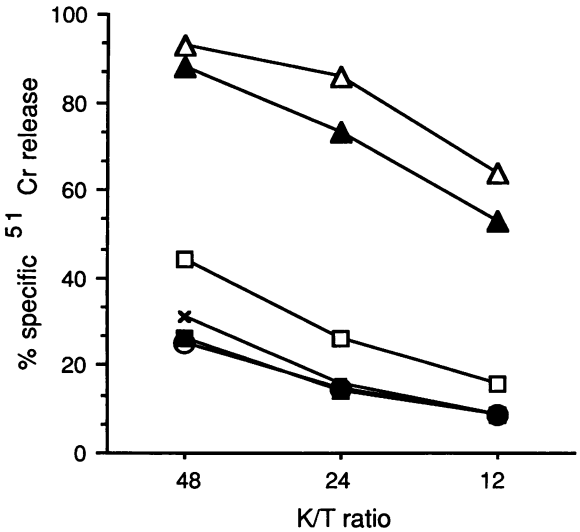

FIG. 2. Target cells expressing the 22K protein are strongly lysed by CTL from mice primed by respiratory tract infection with RS virus. Spleen cells from RS virus-primed BALB/c mice were cultured for 5 days with RS virus-infected stimulator cells (2), and CTL recognition was assayed by infecting P815 cells with RS virus (filled triangle) or with recombinant VV expressing the following proteins: the 22K protein (△); fusion protein (□), phosphoprotein (×), major surface glycoprotein (■), and uninfected control protein (○). Recombinant VV encoding the negative-orientation phosphoprotein or 1a protein were also tested for target formation and not recognized (data not shown).

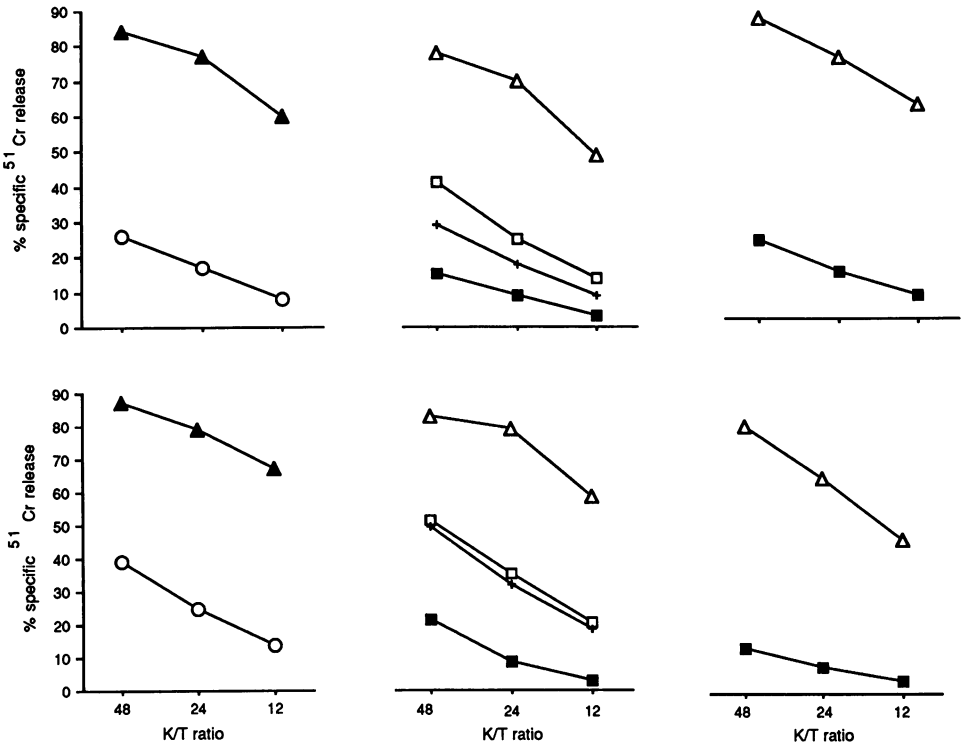

FIG. 3. 22K protein-specific CTL from mice primed by RS virus infection are K^d restricted and show long memory. Spleen cells from BALB/c mice primed 11 weeks (upper graphs) or 11 months (lower graphs) earlier by intranasal infection with RS virus were tested 5 days after in vitro stimulation with RS virus-infected spleen cells. Targets were P815 cells uninfected or infected with RS virus (left panels), various recombinant VV (center panels), or K^d-transfected L929 cells infected with recombinant VV encoding the 22K or G protein (right panels). The symbols are as in Fig. 2, except that + stands for nucleoprotein. Untransfected L929 ($H\text{-}2^k$) cells were not seen, whether infected with RS virus or recombinant VV-22K or uninfected (data not shown).

infected with recombinant VV-22K were lysed, whereas those infected with recombinant VV-G were not, showing that recognition of the 22K protein is K^d restricted and that memory is long lasting. Untransfected L929 cells or cells transfected with D^d and infected with recombinant VV-22K or RS virus were not lysed by BALB/c- or DBA/2-derived CTL (data not shown).

Infection of mice with recombinant VV-22K induces memory CTL for RS virus. To test CTL induction by the recombinant VV, mice were primed by cutaneous scarification with recombinant VV-G, VV-F, VV-P, VV-1A, VV-22K, or wild-type VV. The mice developed typical transient local lesions but did not become ill. After 5 weeks, spleen cells were stimulated with RS virus strain A2-infected cells as described above and tested for lytic ability against P815 cells infected with RS virus or the recombinant VV. As previously shown (34), VV-F primed BALB/c mice strongly for RS virus F protein-specific CTL (40 to 60% specific lysis at a K/T ratio of 24 using CTL from 5-day cultures; data not shown). In contrast, priming with recombinant VV encoding the 22K protein (which is an important target antigen for CTL from RS virus-infected mice; see above) induced relatively low levels of CTL memory cells specific for RS virus (typically, 10 to 15% specific lysis at a K/T ratio of 24; e.g., Fig. 4). To test whether lymphokines might be limiting in the cultures derived from mice primed with recombinant VV-22K, supernatants were exchanged between cultures. There was no increase in killing by cells from mice primed with recombinant VV-22K or reduced killing by cells from those primed with RS virus (data not shown). Priming with recom-

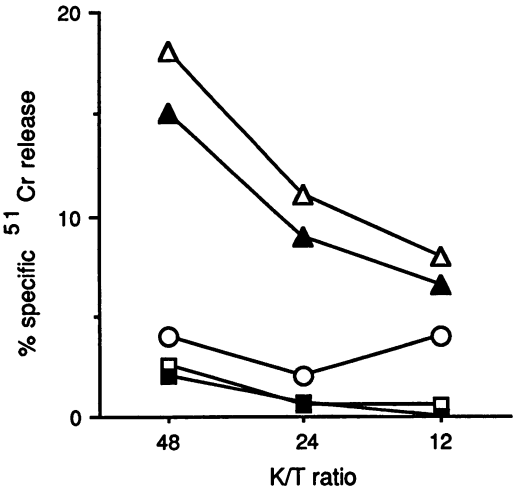

FIG. 4. RS virus-specific CTL generated from mice primed by infection with VV-22K. BALB/c mice were primed with recombinant VV-22K by dermal scarification. After 6 weeks, spleen cells were taken and incubated for 5 days with syngeneic RS virus-infected spleen cells. CTL recognition was tested against P815 cells infected with RS virus (▲), recombinant VV-22K (△), uninfected cells (○), recombinant VV-G (■), or recombinant VV-F (□). Note the change in scale compared with Fig. 2 and 3 and that simultaneously prepared CTL from RS virus-infected mice were much more effective against these same target cells (Fig. 2).

binant VV-P55, VV-1A, or wild-type VV was ineffective in generating detectable RS virus-specific CTL.

DISCUSSION

Human RS viruses contain two proteins with matrixlike properties. In addition to the conventional matrix protein (M_1) found in other paramyxoviruses, they also possess a unique 22-kilodalton matrixlike protein (22K or M_2) of unknown function. Our finding that *H-2*^{d} mice primed by infection of the respiratory tract with RS virus possess memory CTL directed predominantly against this protein could explain the cross-reactive recognition (1) by such CTL. Sequencing of the 22K protein gene from two strains of RS virus isolated 15 years apart in the northern (RSS-2) and southern (A2) hemispheres shows only three amino acid differences, predicting an amino acid homology of 98% (5). These changes are all substitutions of one hydrophobic amino acid by another and may not affect T-cell recognition. The degree of conservation in other serologically dissimilar isolates is unknown.

It has been shown that recombinant VV-F infection is a potent inducer of RS virus-specific memory CTL (34). Surprisingly, infection with recombinant VV-22K also induced RS virus-specific CTL but far less strongly than did priming with recombinant VV-F. This apparent discrepancy with the induction of 22K-specific CTL by intranasal inoculation of RS virus may be due to differences in T_h memory cell induction: priming with recombinant VV-F induces RS virus-specific T_h cells (32), which can presumably help to activate memory CTL in vitro. By contrast, priming with recombinant VV-22K may induce memory CTL without priming for T_h cells; generation of CTL in cultures might then be inefficient (19). In support of this suggestion, preliminary experiments have been performed in which T_h-cell priming was examined by measurement of antigen-induced release of interleukins 2 and 4 from spleen cells of mice primed with recombinant VV. Cytokine release by cells from mice primed with recombinant VV-22K, VV-1a, or wild-type VV was low compared with that from cells from RS virus or recombinant VV-F-primed mice (Openshaw, unpublished data). In experiments in which supernatants were exchanged daily between cultures of spleen cells from mice primed by infection with recombinant VV-22K or RS virus, the CTL activity of VV-22K-infected cells did not increase, indicating that addition of secreted T-cell factors did not amplify memory CTL activation in our cultures. Alternatively, differences in processing and antigen presentation of RS virus native and recombinant antigens and the different sites of priming may cause different antigens to be seen by CTL. It has been suggested that different modes of virus entry into cells may in part determine patterns of T-cell recognition (28), but the exact mechanism of entry of RS virus has not been described.

The 22K protein is of considerable virological interest, having no counterpart in other paramyxoviruses. In salt and detergent dissociation studies, it is membrane associated (22), but it is not displayed on the surface of mature virus (40). It is nonglycosylated and comprises 194 amino acids (5). It is relatively hydrophilic (13) and strongly basic (16). A search for regions of hydrophobicity by the Kyte and Doolittle method (26) showed two short chains of hydrophobic amino acids near the center of the sequence, but these are not of sufficient length to provide a transmembrane component. Secondary structural modeling by previously published methods (12, 18) predicted several alpha-helical motifs joined by turns or coils, typical of a globular protein.

The 22K protein has been immunoprecipitated by using serum from a 5-year-old child recovering from RS virus infection, but antibody responses to it are weak compared with responses to the surface glycoproteins (49). Two mouse monoclonal antibodies to the 22K protein have been found to recognize distinct epitopes (39). Although detected within the cytoplasm of HeLa cells at 17 h after RS virus infection, its surface expression is delayed for a further 16 h. By contrast, F and G are expressed at the surface by 17 h, and P and N do not appear on the surface of infected HeLa cells in serologically detectable quantities (39). Our target cells were lysed by CTL 18 to 20 h after infection, illustrating that CTL can detect antigens when they are not expected to be detectable serologically on the cell surface.

The strong recognition of the 22K protein by virus-specific CTL from *H-2*^{d} mice was not predicted on the basis of studies of other viral systems. CTL from other mouse strains now need testing against our current panel of VV recombinants, but it seems that CTL from C57Bl mice show little or no recognition of VV-F-, VV-N-, or VV-G-infected targets (R. M. Pemberton, P. J. M. Openshaw, and B. A. Askonas, unpublished data). Studies with other viral systems have shown that class I MHC molecules have striking effects on CTL recognition; for example, in influenza virus, the nucleoprotein is recognized in various mouse strains in association with K^d, D^b, or K^k but not with the other class I MHC molecules present in these mice (33). Different class I proteins restrict recognition of different nucleoprotein epitopes (4, 30, 33, 48). Recognition of other internal proteins (NS-1 and polymerases PB1, PB2, and PA) also varies in different strains of mice (7, 8). Lymphocytic choriomeningitis virus-specific CTL from *H-2*^{d}, *H-2*^{b}, and *H-2*^{q} mice all recognize nucleoprotein, but only *H-2*^{b} mice show strong recognition of glycoprotein G (51, 52). Polyclonal CTL from BALB/c (*H-2*^{d}) mice primed with Gross leukemia virus recognize mainly *env* gene products, while BALB/B (*H-2*^{b}) CTL recognize predominantly *gag* products and C57BL/6 (*H-2*^{b}) CTL detect both *env* and *gag* (35). In addition to class I effects, antigen processing and T-cell selection during thymic ontogeny appear to be important in determining CTL recognition patterns (15, 38, 46, 53). It should, however, be noted that identification of a dominant target antigen for CTL induced by viral infection of one site does not necessarily indicate the antigen best able to induce specific CTL recognition of the virus when administered in other forms.

Studies are under way which aim to define the proteins seen by CTL from human donors. We hope to explore the relationship of MHC gene products to RS virus protein recognition and the relationship of recognition patterns to virus clearance and immunopathologic changes of RS virus infections.

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