JOURNAL OF VIROLOGY, Sept. 1990, p. 4232–4241
0022-538X/90/094232-10$02.00/0

Vol. 64, No. 9

Cytolytic T-Lymphocyte Responses to Respiratory Syncytial Virus: Effector Cell Phenotype and Target Proteins

J. A. NICHOLAS,[1]* K. L. RUBINO,[1] M. E. LEVELY,[1] E. G. ADAMS,[2] AND P. L. COLLINS[3]

Departments of Infectious Diseases[1] and Cancer Research,[2] The Upjohn Company, Kalamazoo, Michigan 49007, and Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[3]

Received 26 March 1990/Accepted 31 May 1990

Cytolytic T-lymphocyte (CTL) activity specific for respiratory syncytial (RS) virus was investigated after intranasal infection of mice with RS virus, after intraperitoneal infection of mice with a recombinant vaccinia virus expressing the F glycoprotein, and after intramuscular vaccination of mice with Formalin-inactivated RS virus or a chimeric glycoprotein, FG, expressed from a recombinant baculovirus. Spleen cell cultures from mice previously infected with live RS virus or the F-protein recombinant vaccinia virus had significant CTL activity after one cycle of in vitro restimulation with RS virus, and lytic activity was derived from a major histocompatibility complex-restricted, Lyt2.2$^+$ (CD8$^+$) subset. CTL activity was not restimulated in spleen cells from mice that received either the Formalin-inactivated RS virus or the purified glycoprotein, FG. The protein target structures for recognition by murine CD8$^+$ CTL were identified by using target cells infected with recombinant vaccinia viruses that individually express seven structural proteins of RS virus. Quantitation of cytolytic activity against cells expressing each target structure suggested that 22K was the major target protein for CD8$^+$ CTL, equivalent to recognition of cells infected with RS virus, followed by intermediate recognition of F or N, slight recognition of P, and no recognition of G, SH, or M. Repeated stimulation of murine CTL with RS virus resulted in outgrowth of CD4$^+$ CTL which, over time, became the exclusive subset in culture. Murine CD4$^+$ CTL were highly cytolytic for RS virus-infected cells, but they did not recognize target cells infected with any of the recombinant vaccinia viruses expressing the seven RS virus structural proteins. Finally, the CTL response in peripheral blood mononuclear cells of adult human volunteers was investigated. The detection of significant levels of RS virus-specific cytolytic activity in these cells was dependent on at least two restimulations with RS virus in vitro, and cytolytic activity was derived primarily from the CD4$^+$ subset.

Respiratory syncytial (RS) virus is a member of the family *Paramyxoviridae* and is the leading viral etiologic agent of severe bronchiolitis and pneumonia in infants (8, 17). Its negatively stranded RNA genome encodes 10 proteins detectable in virus-infected cells. At least seven of these proteins appear to be virion structural components: three nucleocapsid-associated proteins, N, P, and L; two nonglycosylated internal membrane-associated proteins, M and 22K (also called M2); and two transmembrane surface glycoproteins, G and F. An additional protein, SH (also called 1A), was recently shown to be a transmembrane protein that is present in both glycosylated and nonglycosylated forms at the surface of infected cells (37) and appears to be an eighth virion structural protein (20; P. L. Collins, R. A. Olmsted, and P. R. Johnson, J. Gen. Virol., in press). The remaining two proteins, 1C and 1B (also called NS1 and NS2, respectively) appear to be nonstructural species (20).

Repeated infection with RS virus may occur throughout life, although the severity of disease becomes substantially diminished upon third reinfection in childhood (14, 19). Although there have been numerous studies of humoral and cellular immunity to RS virus in humans and in experimental animals, the immune mechanisms that underlie the balance between protection, disease, and recovery remain poorly understood. The most striking upset of this balance occurred after administration of a Formalin-inactivated whole-virus vaccine to infants: the vaccine induced neutralizing and complement-fixing antibodies (10, 16, 27, 28) and augmented the antigen-specific lymphocyte transformation response in vaccinees (29), yet it was not effective in preventing subsequent natural infection. Furthermore, upon natural exposure to RS virus, vaccinees suffered increased frequency of hospitalization and enhanced disease compared with children who received a control immunization (10, 16, 27, 28). Experimental administration of Formalin-inactivated RS virus, followed by infection, is associated with enhanced pulmonary histopathology in cotton rats, albeit without clinical disease (36, 40). Another striking example of disease enhancement was reported in a mouse model of RS virus infection in which passive transfer of cytolytic T lymphocytes (CTL) rapidly cleared virus from the lung but also intensified clinical symptoms, enhanced pulmonary pathology, and increased death rates (6). Alternatively, other observations suggest a role for CTL in protective immunity to RS virus. For example, Taylor et al. (44) reported that the natural occurrence of the CTL response after intranasal RS virus infection of mice correlated with virus clearance and recovery. RS virus-specific CTL develop in response to natural infection of humans (3, 9, 21). Infants under 6 months of age are at greatest risk of severe RS virus infection, and it has been reported that infants under 3 months of age exhibit CTL responses for RS virus with low frequency (9, 21). Moreover, CTL responses are more readily detected in older children, in whom it has been suggested that repeated infection may contribute to this response (9). Investigations of the major histocompatibility complex (MHC) class I-restricted CTL response of mice (2, 39) and humans (2) to RS virus have defined the N and F proteins to be primary

* Corresponding author.

targets. Further characterization of the RS virus-specific CTL responses in experimental animals and humans is needed for identifying factors that may be important to the balance between protective immunity and disease enhancement.

In this report, we compared the murine CTL response to live versus nonreplicating RS virus preparations. After live virus infection, we distinguished four target proteins for murine $CD8^+$, MHC class I-restricted CTL by using recombinant vaccinia viruses. To better understand the impact of repeated reinfection on the CTL response, we used an in vitro correlate for repeated antigen stimulation of murine and human CTL by live RS virus to investigate the effect on CTL phenotype and target protein recognition.

MATERIALS AND METHODS

Viruses and cells. RS virus strains A2 and Long were grown in HEp-2 cells. Titers of RS virus were determined by plaque assay on HEp-2 cells under 0.5% agarose containing Eagle minimal essential medium (Whittaker Bioproducts) and 3% fetal bovine serum (FBS; GIBCO Laboratories). The A2 and Long strains have been shown to be very closely related by antigenic and sequence analyses (see references 1, 25, and 30 and references therein). Recombinant vaccinia viruses expressing the F, G, and SH proteins of RS virus were previously described (13, 37, 38), as was the vaccinia virus recombinant expressing the pseudorabies virus gp50 (31). Recombinant vaccinia viruses were grown in Vero cells or in HEp-2 cells and titrated under 0.5% agarose containing Eagle minimal essential medium with 3% FBS. Recombinant vaccinia viruses expressing M, P, and 22K proteins were constructed by using previously described cDNAs (12, 42, 43). cDNA encoding the N protein was resynthesized and recloned, using the published sequence (11) as a guide to obtain the desired cDNA (unpublished data). Noncoding regions of the RS virus genes were removed as follows. Each cDNA was modified by site-directed mutagenesis to contain a *Bam*HI restriction site at positions −9 to −4 relative to the translational start site of the open reading frame (ORF). The N and M cDNAs were similarly modified to each contain a *Bam*HI site at positions 4 and 9 relative to the translational stop site of the ORF. For the 22K or P gene, respectively, a preexisting *Bam*HI (nucleotides 904 to 909) or *Hpa*II (nucleotides 880 to 884) site downstream of the end of the ORF was used for removing noncoding sequences. The cDNAs were placed under the control of the early-late P7.5 vaccinia virus promoter and inserted into the thymidine kinase locus of the WR strain of vaccinia virus, using the β-galactosidase coexpression method (7) to identify recombinant viruses. The identification of recombinants was confirmed by restriction analysis and Southern blot hybridization, and expression of the correct RS virus protein by independent isolates was confirmed as described below. The same methods, vectors, and vaccinia virus strain had been used to construct the recombinant vaccinia viruses expressing the F, G, and SH proteins. Formalin-inactivated RS virus of the Long strain was prepared in serum-free medium as described previously (40). This preparation was without detectable residual infectivity and was associated with enhanced lung histopathology when immunized cotton rats were challenged with RS virus (M. Wathen, unpublished observations) as described previously (40). The FG chimeric protein was modeled on the A2 strain of RS virus and purified from a baculovirus expression system as previously described (46). Each of these preparations was precipitated with aluminum hydroxide (Alhydrogel; Superfos), resulting in a 100× concentration of the Formalin-inactivated RS virus, for intramuscular immunization as described previously (5); these preparations were a generous gift of Michael Wathen. BALB/c and BCH_4 cells were a kind gift of Bruce Fernie and were described previously (15). BALB/c cells represent uninfected control $H\text{-}2^d$ cells, whereas BCH_4 cells are a derivative of BALB/c cells persistently infected with the Long strain of RS virus. L929 cells ($H\text{-}2^k$) were obtained from the American Type Culture Collection, Rockville, Md. A20-1.11 cells, a B-cell lymphoma expressing both class I MHC ($H\text{-}2^d$) and class II MHC ($IA^d\ IE^d$) molecules, were a kind gift of Tom Braciale. Transformation of human B lymphocytes with Epstein-Barr virus from B95-8 marmoset cells was done as described previously (22).

CTL priming and restimulations. BALB/c mice at least 6 weeks old were anesthetized with sodium pentobarbital and ketamine, and $10^{5.5}$ to 10^6 PFU of RS virus, strain A2 or Long, was delivered intranasally. Recombinant vaccinia viruses (10^6 PFU) were given intraperitoneally. Alum-precipitated, Formalin-inactivated RS virus (5 or 25 μl) or purified FG glycoprotein (0.2 or 1.0 μg) was given intramuscularly two times 2 weeks apart. At least 3 weeks after immunization or infection, spleen cells of three to five mice were pooled for stimulation of memory CTL activity. Memory immune spleen cells were cultured at 2×10^6 cells per ml with the homologous strain of live RS virus at a multiplicity of infection (MOI) of 0.1 to 1.0 in RPMI 1640 medium (Whittaker) containing 10% FBS, 2 mM L-glutamine (Whittaker), 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (Biologos), 5×10^{-5} M 2-mercaptoethanol (Bio-Rad Laboratories), and 50 μg of gentamicin sulfate per ml (MLR medium). CTL activity of these secondary cultures was assayed as described below. For continuous, weekly restimulation of immune CTL, viable cells were purified by centrifugation on Ficoll (Pharmacia) and subcultured at 3×10^5 to 7×10^5 cells per ml in MLR medium with 10^6 gamma-irradiated, RS virus-infected (MOI = 0.1 to 1.0) feeder spleen cells derived from naive BALB/c mice. Cultures were supplemented with 10% rat interleukin-2 prepared from concanavalin A-stimulated spleen cells as described previously (47), and the lectin was inactivated by addition of 0.5 M methyl-α-D-mannopyranoside (Sigma Chemical Co.). CTL activity of immune spleen cells was typically tested after 5 to 6 days of virus stimulation in vitro. For human CTL stimulation, peripheral blood mononuclear cells (PBMC) were derived from normal adult donors via leukapheresis and purification on Ficoll (Pharmacia). Samples of PBMC were stored frozen in liquid nitrogen until use. For initial CTL stimulation in vitro, 10^6 PBMC per ml were cultured in MLR medium (substituting 10% human AB serum [Hazleton Laboratories] for FBS) with RS virus at MOI = 1.0. These CTL were restimulated by weekly addition of autologous, gamma-irradiated PBMC (10^6 per stimulation) infected with RS virus (strain A2) at MOI = 0.5 to 1.0 and supplemented with 10 to 20% human interleukin-2 (PanData). Typically, these cultures were subjected to Ficoll purification of viable cells every other week. CTL activity of human cells was typically tested after 8 days of stimulation.

Target cell preparation and CTL assay. Target cells, either Epstein-Barr virus-transformed B lymphocytes, A20-1.11 cells, or L929 cells, were infected at MOI = 5 to 10 with RS virus or with recombinant vaccinia viruses approximately 16 to 24 h before assay. Adherent target cells, either L929, BALB/c, or BCH_4 cells, were removed with trypsin-EDTA before labeling. All target cells were labeled in suspension

with 100 to 200 μCi of ^{51}Na-chromate (Dupont) for 1 to 2 h at 37°C in medium containing 10% FBS, followed by extensive washing in medium containing 2% FBS. Target cells (10^4) were added to effector CTL in microtiter plates in MLR medium, centrifuged, and incubated for 4 h at 37°C. Half of the supernatant volume (100 μl) was assayed for ^{51}Cr release in a Packard gamma scintillation spectrophotometer. The percent specific ^{51}Cr release was calculated as {[counts per minute released by effector cells − counts per minute released in medium]/[total counts per minute released by detergent (3% Triton X-100) − counts per minute released in medium]} × 100.

Subset depletion studies. Mouse spleen cells or CTL (10^7) were reacted with 1 ml of medium, 1 ml of supernatant fluid GK1.5 (anti-L3T4, immunoglublin G [IgG]), or 1 ml of a 1:50 dilution of ascites fluid AD_4(15) (anti-Lyt2.2, IgM) in the presence of rabbit complement (1:14; Low-Tox-M; Cedarlane) for 60 min at 37°C. This concentration of complement had no effect on CTL activity or flow cytometric analyses in the absence of subset-specific antibody. Cells were washed twice in MLR medium before CTL assay or flow cytometry. Effector/target ratios were based on viable cell counts before complement depletion. Human CTL (2×10^6 per reaction) were incubated with medium alone, with a combination of two mouse monoclonal antibodies to two different determinants of CD4 (IgG3 and IgG2a, respectively), or with a mouse monoclonal antibody to CD8 (IgG1) for 60 min at 4°C. These monoclonal antibodies were a generous gift of Jefferson Paslay. The cells were washed three times in phosphate-buffered saline containing 10% FBS and suspended with 100 μl of magnetic beads (Dynal, Bioproducts for Science) or magnetic particles (BioMag; Advanced Magnetics Inc.) precoated with sheep or goat anti-mouse IgG for 45 min at 4°C. The reaction mixture was diluted 30-fold in phosphate-buffered saline with 10% FBS, and magnetic particles were removed with a magnetic particle separator (MPC-1; Dynal). Cells not bound to beads or particles retained by the magnet were collected and used for CTL assay or flow cytometric analysis. Effector/target ratios were based on the original, nondepleted cell number. The success of the depletion protocol was quantitated by flow cytometry.

Flow cytometry. Mouse spleen cells or CTL were stained with fluorescein isothiocyanate-labeled antibody to Lyt2 and phycoerythrin-labeled antibody to L3T4 (both from Becton Dickinson Immunocytochemistry Systems) as specified by the manufacturer (10^6 cells per 10 μg of antibody). Human PBMC or CTL were stained with the Simultest kit (Becton Dickinson) as directed by the manufacturer (see above). Stained cells were washed in phosphate-buffered saline and fixed in 2% paraformaldehyde. Samples were analyzed for immunofluorescence staining on a FACStarPLUS flow cytometer (Becton Dickinson Immunocytometry Systems [BDIS]). Illumination was at a laser wavelength of 488 nm and power of 200 mW. Fluorescein fluorescence was collected through a DF 530/30-nm three-cavity filter (BDIS no. 2487). Phycoerythrin fluorescence was collected through a DF 575/26-nm three-cavity filter (BDIS no. 1687). Forward and wide-angle laser light scatter were collected through a BP 488/10-nm filter (BDIS no. 2887). All data were acquired in list mode on a MicroVAX-II computer. Where appropriate, list mode data were edited by using forward-versus-side laser light scatter gates to select lymphocytic cells for analysis.

RESULTS

Induction of RS virus-specific CD8$^+$ CTL. Our initial studies investigated the induction of CTL activity after intranasal infection of mice with live RS virus or intraperitoneal infection with a recombinant vaccinia virus expressing the F glycoprotein. Both the A2 and Long strains of RS virus established pulmonary infection in BALB/c mice, as evidenced by recovery of infectious virus from lung homogenates. Typical virus titers 4 days postinfection were 2×10^3 to 8×10^3 PFU/g of lung tissue after A2 virus infection and 2×10^4 to 3×10^4 PFU/g of lung tissue after Long virus infection. Three or more weeks after live virus inoculation or vaccination, memory spleen cells were stimulated once in vitro with either the A2 or Long strain of virus, and cytolytic activity was assayed 5 days later (Fig. 1). High levels of cytolytic activity for persistently infected BCH_4 cells were detected in secondary cultures derived from mice infected with either the A2 or Long strain or RS virus, confirming a high degree of relatedness of these two virus strains (1), or from mice infected with the recombinant vaccinia virus expressing the F glycoprotein (Fig. 1). Spleen cells from all three groups of mice had negligible cytolytic activity for uninfected BALB/c cells, for uninfected, histoincompatible L929 cells, or for L929 cells infected with RS virus (Fig. 1). The inability to lyse infected L929 cells was not a result of lack of expression of RS viral antigens, since flow cytometric analysis of cells treated with fluorescein isothiocyanate-labeled antiserum to RS virus revealed high levels of surface viral antigens during the time corresponding to that of the assay (data not shown). Finally, cytolytic activity on BCH_4 cells was reduced by 85% to more than 90% when effector cells were pretreated with antibody to Lyt2.2 plus complement (Fig. 1). Together, these data establish that the predominant cytolytic activity induced by primary infection of mice with either the A2 or Long strain of RS virus or the F-protein recombinant vaccinia virus was derived from virus-specific, MHC-restricted, Lyt2$^+$ CTL, hereafter referred to as CD8$^+$ CTL.

Formalin-inactivated RS virus or purified FG glycoprotein did not induce CTL activity. We next determined whether CTL activity could be induced by intramuscular immunization of mice with alum-precipitated vaccines compared with live virus inoculation. For these studies, cytolytic activity was measured on cells expressing only class I MHC determinants (BCH_4 cells) and on the B-lymphoma cell line, A20-1.11, which also expresses class II MHC determinants. The rationale for using both types of target cells was to distinguish CD8$^+$ CTL from CD4$^+$ CTL, since importance has been ascribed to the latter when inactivated virus preparations are used (33, 35). A Formalin-inactivated RS virus vaccine was prepared and used for immunization in a manner analogous to that used for the original vaccine (10, 16, 27–29) and as previously reported by others (36, 40). The purified glycoprotein vaccine, FG, was previously described (46) and shown to fully protect cotton rats from live virus challenge (5). No cytolytic activity was measurable in spleen cell cultures derived from mice immunized with Formalin-inactivated RS virus or from mice immunized with the FG glycoprotein (Fig. 2) regardless of the class of MHC expressed by the target cells. However, both vaccines induced high levels of RS virus-specific antibody (5; data not shown). Live virus inoculation again induced high levels of cytolytic activity measurable on both BCH_4 cells and RS virus-infected A20-1.11 cells (Fig. 2). Treatment of effector cells with antibody to CD4 (anti-L3T4) and complement had

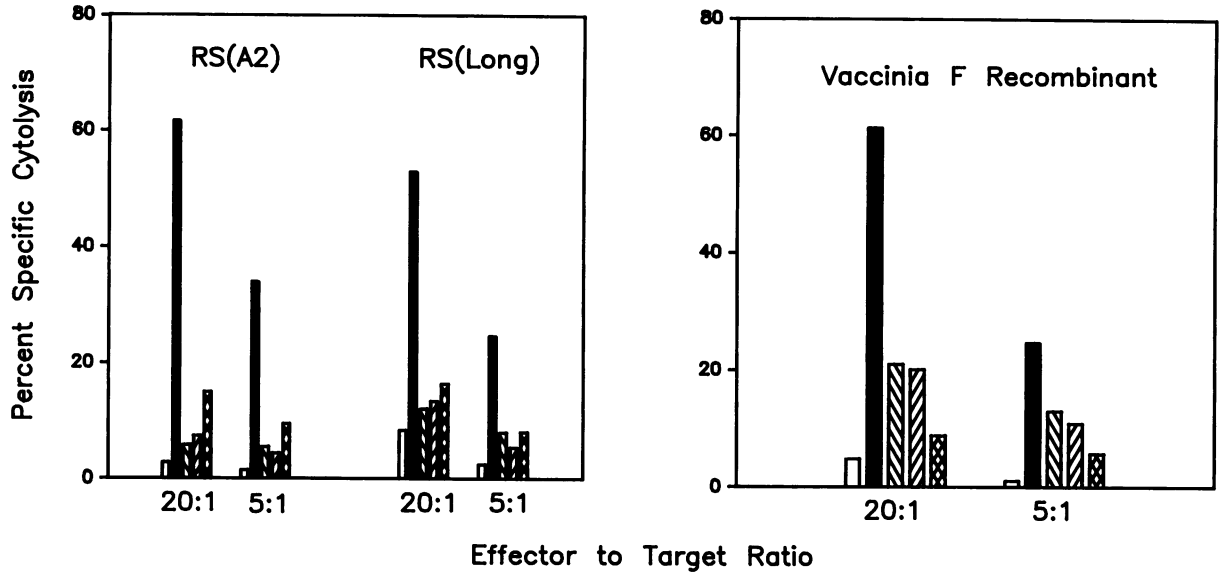

FIG. 1. CTL induced by RS virus or by a recombinant vaccinia virus expressing the RS viral F protein: MHC restriction and $CD8^+$ phenotype after secondary stimulation in vitro. CTL were induced in BALB/c mice by intranasal instillation of the indicated strain of RS virus or by intraperitoneal inoculation of a recombinant vaccinia virus expressing the RS viral F glycoprotein. Three or more weeks later, spleen cells were restimulated once in vitro, and CTL activity was assayed. Symbols: □, lysis of BALB/c uninfected fibroblasts; ■, lysis of histocompatible BCH_4 fibroblasts persistently infected with RS virus strain Long; ▨, lysis of histoincompatible L929 cells infected with the homologous RS virus strain; ▩, lysis of uninfected L929 cells; ▩, lysis of BCH_4 fibroblasts persistently infected with RS virus strain Long after pretreatment of CTL with anti-Lyt2.2 monoclonal antibody [AD_4(15)] and complement. Data are representative of at least three independent experiments.

negligible, if any, effect on cytolytic activity against target cells bearing class II MHC molecules, whereas treatment with antibody to CD8 (anti-Lyt2.2) and complement fully abolished cytolytic activity against infected A20-1.11 cells (Fig. 2). These studies confirmed that murine CTL activity after one or two stimulations in vitro was predominantly within the $CD8^+$ subset.

Target protein specificities for murine $CD8^+$ CTL. The RS virus target protein specificity of $CD8^+$ CTL derived after one or two stimulations in vitro was investigated in A20-1.11 cells infected with recombinant vaccinia viruses expressing individual proteins of RS virus compared with that of A20-1.11 cells infected with RS virus or with a recombinant vaccinia virus expressing the major glycoprotein of pseudorabies virus, gp50 (Fig. 3). The expression of each of these proteins is driven by the early-late P7.5 promoter inserted into the thymidine kinase locus of vaccinia virus. The dominant target antigen for $CD8^+$ CTL was the 22K protein, and recognition of cells expressing this target structure was quantitatively similar to recognition of cells infected with live RS virus (Fig. 3). RS virus-induced CTL also lysed A20-1.11 cells infected with recombinant vaccinia viruses expressing F or N but not SH, G, or M (Fig. 3). We also observed low but reproducible cytolysis of target cells expressing P (Fig. 3). It was possible that failure of CTL to recognize some of these target structures was a consequence of inadequate expression of the viral protein. However, expression of high levels of antigenically authentic RS viral G, F, and SH proteins by the corresponding recombinant vaccinia viruses was shown in previous work (13, 37, 38), and the expression of authentic N, M, P, and 22K proteins by the corresponding vaccinia viruses was herein confirmed by immunoprecipitation (Fig. 4). For these studies, postinfection cotton rat serum was used to immunoprecipitate the corresponding N, M, P, or 22K protein under conditions of antibody excess, and results (Fig. 4) suggested that the recombinant vaccinia viruses synthesized levels of antigenically authentic protein equal to levels of protein expression observed in RS virus-infected cells.

Detection of murine $CD4^+$ CTL after multiple antigenic stimulations. Since RS virus repeatedly infects humans thoughout life (14, 19), albeit with increasingly milder episodes after the second or third infection, it was of interest to determine the effects of repeated exposure of spleen CTL to RS virus in vitro. During the course of weekly restimulation of RS strain A2 virus-induced CTL, we periodically analyzed the cultures for surface phenotype and lytic activity (Fig. 5). After one to three stimulations, cells of the $CD8^+$ phenotype predominated (Fig. 1, 2, and 5a); as restimulation continued, a mixture of $CD4^+$ and $CD8^+$ cells was found (Fig. 5a), both subsets of which had cytolytic activity for RS virus-infected A20-1.11 cells (Fig. 5b), as determined by depleting individual subsets with monoclonal antibody and complement. As reexposure to RS virus continued, $CD4^+$ CTL became the predominant, if not exclusive, subset in culture (Fig. 5a). The $CD4^+$ CTL lines retained potent cytolytic activity for A20-1.11 cells infected with RS virus (Fig. 6) but failed to lyse BCH_4 cells (data not shown), consistent with class II MHC restriction, and they did not lyse RS virus-infected target cells expressing xenogeneic class II MHC determinants (data not shown).

Although the $CD4^+$ CTL lines were highly cytolytic for RS virus-infected A20-1.11 cells, surprisingly, they did not lyse A20-1.11 target cells infected by any of our panel of recombinant vaccinia viruses (data not shown). Three $CD4^+$ CTL lines were independently established, and all three failed to recognize A20-1.11 cells infected with any of our recombinant vaccinia viruses.

Human RS virus-specific CTL are found in the $CD4^+$ subset. It was of interest to determine the relevance of $CD4^+$

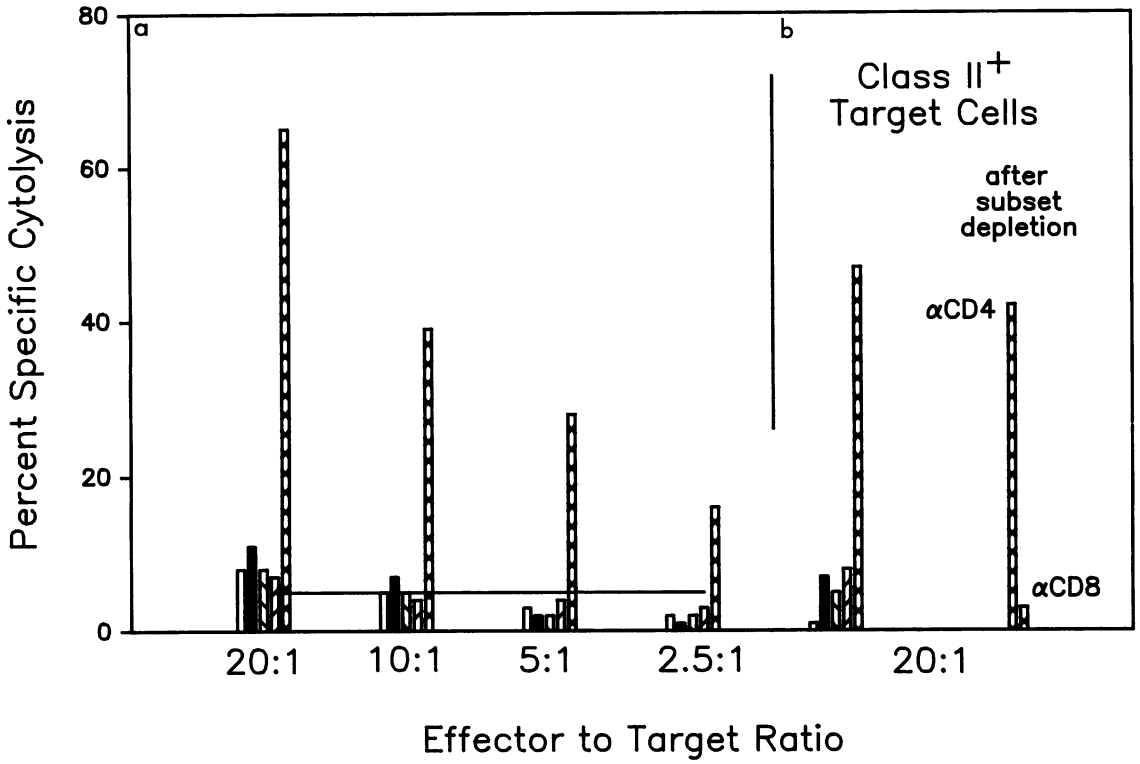

FIG. 2. Lack of induction of CTL by vaccination with Formalin-inactivated RS virus or a purified, chimeric FG glycoprotein. BALB/c mice were twice vaccinated intramuscularly at 2-week intervals with Formalin-treated RS virus (Long strain) or with FG glycoprotein (A2 strain) formulated in alum, or mice received a single dose of live RS (Long strain) virus by intranasal instillation. At least 3 weeks after final vaccination, spleen cells were restimulated once in vitro with RS virus, and CTL activity was assayed against persistently infected BCH_4 fibroblasts (a) or against A20-1.11 B-lymphoma cells infected with RS virus (b). The horizontal line represents the mean lytic activity among all five groups of uninfected BALB/c fibroblasts. Cytolysis on uninfected A20-1.11 cells was subtracted from cytolysis on A20-1.11 cells infected with RS virus and represents net cytolysis. Symbols: □ and ■, lytic activity after vaccination with 25 and 5 μl of Formalin-inactivated RS virus, respectively; ▨ and ▩, lytic activity after vaccination with 1.0 and 0.2 μg of FG glycoprotein, respectively; ▩, CTL activity after live virus instillation (before or after T-cell subset depletion). Lysis of RS virus-infected, allogeneic L929 cells was less than 12% at an effector/target ratio of 20:1 and less than 4% at all other ratios.

and $CD8^+$ CTL activity defined above in mice to CTL activity that could be detected in PBMC of adult humans who, presumably, have had repeated exposure to natural RS virus infection throughout life. PBMC from several normal adult donors were prescreened for proliferative activity after a single in vitro stimulation by RS virus strain A2, and one donor was selected from our panel for further study. Epstein-Barr virus-transformed B-lymphoblastoid cell lines from this and one other donor were established to serve as histocompatible and histoincompatible target cells. After at least two restimulations in vitro, human PBMC had significant cytolytic activity for histocompatible B-lymphoblastoid cell lines infected with RS virus (Fig. 7a). After three to five stimulations in vitro, these human CTL lines were depleted of $CD8^+$ cells (greater than 98% depletion as determined by flow cytometry) or depleted of $CD4^+$ cells (approximately 80% depletion as determined by flow cytometry), and residual cytolytic activity was measured on RS virus-infected B-lymphoblastoid cells (Fig. 7a). Depletion of $CD8^+$ cells slightly reduced the cytolytic activity of the CTL line, whereas depletion of $CD4^+$ cells from the effector population reduced cytolytic activity to background levels (Fig. 7a). Phenotypic analysis of CTL after continued reexposure to RS virus revealed that the predominant, if not exclusive, subset within the population was $CD4^+$ after as few as five stimulations in vitro (Fig. 7b).

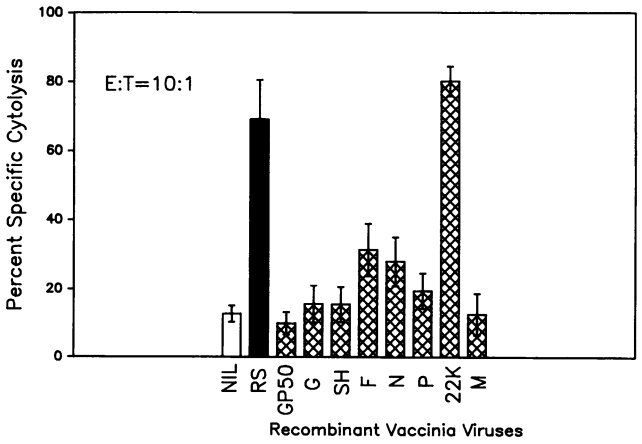

FIG. 3. Target protein specificities of RS virus-induced CTL identified with recombinant vaccinia viruses. Splenic CTL were induced and restimulated once or twice in vitro with either the A2 or Long strain of RS virus. CTL activity was assayed on uninfected A20-1.11 lymphoma cells (□), on RS virus-infected A20-1.11 cells (■), or on A20-1.11 cells infected with recombinant vaccinia viruses (▩) expressing a control protein from pseudorabies virus (gp50) or one of the structural proteins of RS virus. Data represent the means and standard deviations of three independent experiments.

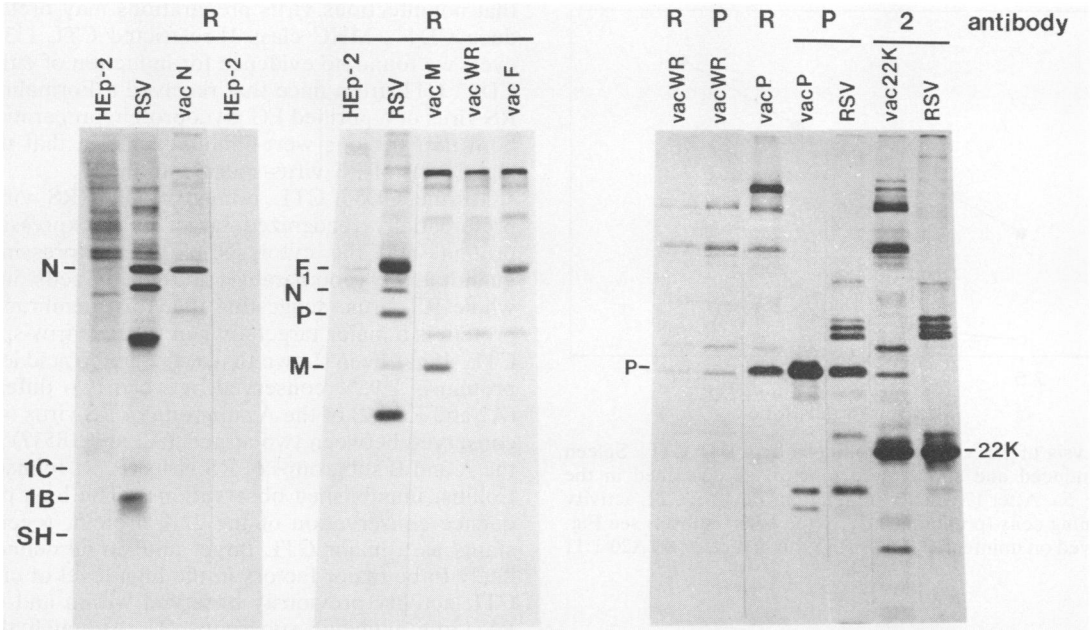

FIG. 4. Expression of authentic RS virus N, P, M, and 22K proteins by recombinant vaccinia viruses. Monolayer cultures of HEp-2 cells were mock infected (HEp-2) or were infected (MOI = 5) with RS virus (RSV), with parental strain WR vaccinia virus (WR), or with a recombinant vaccinia virus expressing the N (vacN), P (vacP), M (vacM), 22K (vac22K), or F (vacF) protein. The cells were labeled by incubation with [^{35}S]methionine for 1 h at 20 h postinfection for the RS virus-infected cells or at 10 h postinfection for the vaccinia virus-infected cells. The cells were lysed in immunoprecipitation buffer (37), clarified by centrifugation at 15,000 × *g* for 5 min, and analyzed by immunoprecipitation with antiserum from cotton rats that had been immunized by three rounds of intranasal RS virus infection at 3-week intervals (R) or with a monoclonal antibody specific to the P (P) or 22K (2) protein. The cotton rat antiserum was used under conditions of antibody excess and was followed by the addition of an excess of rabbit antiserum raised against immunoaffinity gel-purified cotton rat IgG. Antigen-antibody complexes were collected with Sephadex beads containing *Staphylococcus aureus* A protein (Pharmacia) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The cotton rat and rabbit sera were the generous gifts of Gregory A. Prince, and the monoclonal antibodies to P and 22K were the generous gifts of R. Michael Hendry. The positions of the N, M, P, and 22K proteins and the F_1 subunit of the F protein are indicated.

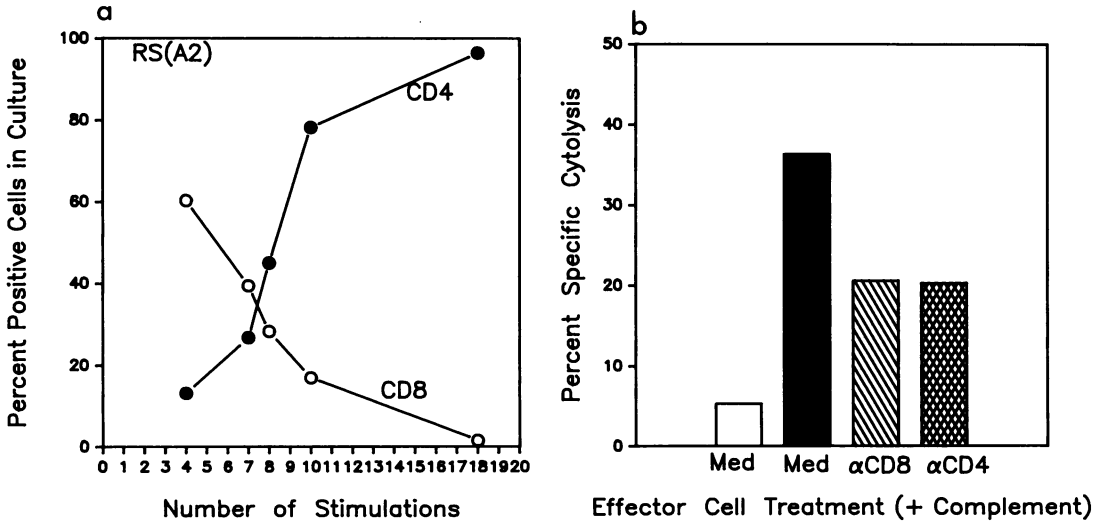

FIG. 5. Phenotypic and cytolytic profile of CTL upon multiple exposures to RS virus strain A2 in vitro. (a) CTL were induced in BALB/c mice by intranasal instillation of the A2 strain of RS virus. Splenic CTL were restimulated weekly by subculture on virus-infected, irradiated feeder cells derived from the spleens of naive mice. Stimulation 1 refers to the in vivo CTL-priming exposure. At the indicated times, CTL were stained with monoclonal antibody to CD4 (phycoerythrin-labeled anti-L3T4) or CD8 (fluorescein isothiocyanate-labeled anti-Lyt2) and analyzed by dual laser flow cytometry. Data are representative of three independent experiments. (b) After eight stimulations, RS virus strain A2-induced CTL were treated with medium (Med), with anti-Lyt2.2 (αCD8), or with anti-L3T4 (αCD4) and complement as described in Materials and Methods. Residual cytolytic activity was assayed on uninfected (□) or RS virus-infected (■, ▨, and ▩) A20-1.11 cells.

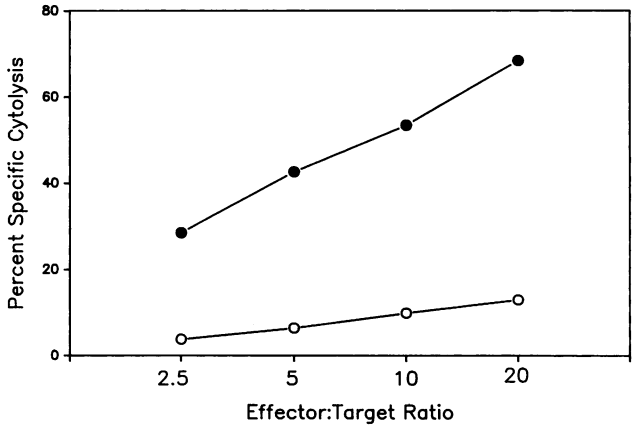

FIG. 6. Lysis of RS virus-infected cells by CD4$^+$ CTL. Spleen CTL were induced and subcultured in vitro as described in the legend to Fig. 5a. After 17 weekly stimulations in vitro, CTL activity of the remaining cells (predominantly of the CD4$^+$ subset; see Fig. 5a) was assayed on uninfected (○) or RS virus-infected (●) A20-1.11 cells.

DISCUSSION

In these studies, MHC-restricted, Lyt2.2$^+$ (CD8$^+$) CTL activity was readily restimulated from memory spleen cells derived from mice infected with live RS virus or an F-protein recombinant vaccinia virus. We also evaluated the ability of a Formalin-inactivated RS virus preparation or a purified, chimeric glycoprotein, FG, to prime murine CTL in vivo, since CTL activity has been implicated in disease enhancement upon RS virus infection in a murine model (6) and previously a Formalin-inactivated vaccine for RS virus appeared to enhance disease in humans upon natural exposure to virus (10, 16, 27–29). For these studies, we kept in mind that noninfectious virus preparations may preferentially induce CD4$^+$, MHC class II-restricted CTL (33, 34). However, we found no evidence for induction of either CD8$^+$ or CD4$^+$ CTL from mice that received a Formalin-inactivated RS virus or a purified FG glycoprotein preparation, although both preparations were immunogenic in that they induced high levels of RS virus-specific antibody.

Murine CD8$^+$ CTL primed by live RS virus infection preferentially recognized target cells expressing the 22K protein, and the cytolysis of cells expressing 22K was quantitatively comparable to that of cells infected with whole RS virus, suggesting that this membrane-associated protein is a major target antigen for RS virus-specific CD8$^+$ CTL. It has been shown that at the amino acid level, the 22K protein is >95% conserved between two different isolates (A2 and RSS-2) of the A subgroup of RS virus (4) and >93% conserved between two strains (A2 and 18537) representing the A and B subgroups of RS virus (P. R. Johnson and P. L. Collins, unpublished observations). The high degree of sequence conservation of the 22K protein, together with its status as a major CTL target antigen as defined here, are likely to be major factors in the high level of cross-reactive CTL activity previously observed within and between the RS virus antigenic subgroups (1). Although the N and F proteins are also well conserved between the antigenic subgroups of RS virus (reference 25 and 30 and references therein), their status as secondary CTL targets as defined here would suggest that they are of subordinate importance in cross-reactive CTL activity. These considerations may prove important for formulating the antigen content of subunit RS virus vaccines. The identification of the 22K protein as a major target antigen for murine CD8$^+$ CTL is offered with the caveat that the 22K gene contains two partially overlapping translational ORFs (12). The 22K protein appears to be encoded by the first ORF, whereas no protein product has yet been identified as corresponding to

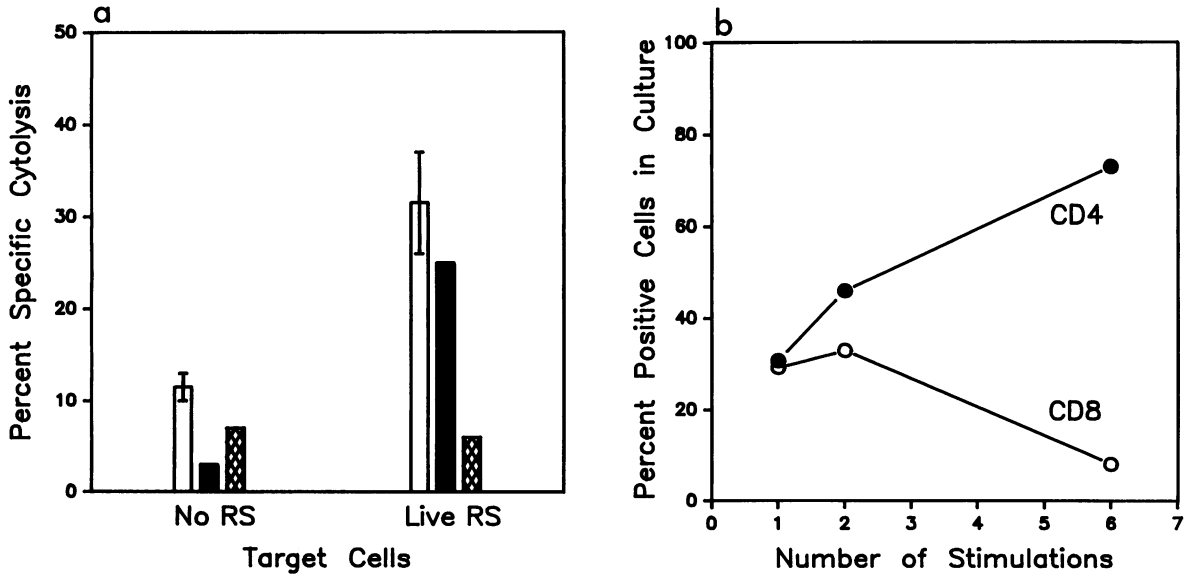

FIG. 7. Cytolytic activity of the CD4$^+$ and CD8$^+$ subsets and phenotypic profile of human peripheral blood CTL induced by multiple stimulations with RS virus. (a) Human CTL were stimulated in vitro three to five times with RS virus strain A2 and assayed for cytolytic activity on autologous Epstein-Barr virus-transformed B lymphocytes that were uninfected or infected with live RS virus. Symbols: □, cytolytic activity of the unseparated lymphocyte population; ■, cytolytic activity of cells after removal of the CD8$^+$ subset; ▩, cytolytic activity of cells after removal of the CD4$^+$ subset. (b) After repeated stimulation of human PBMC with RS virus strain A2 in vitro, cells were analyzed by flow cytometry for surface phenotype. Stimulation 1 refers to freshly isolated cells before in vitro restimulation.

the second ORF. Nonetheless, the possibility exists that this second ORF is expressed, perhaps with low efficiency, by internal ribosomal initiation or by frame shifting at the transcriptional or translational level. We are currently constructing recombinant vaccinia viruses containing each ORF to address this issue.

RS virus-induced $CD8^+$ CTL also consistently and significantly lysed target cells expressing either the F or N protein but not the G, SH, or M protein. These data support and extend the observations of Bangham et al. (2), who reported that RS virus-induced, MHC class I-restricted CTL of both humans and mice recognized target cells infected with recombinant vaccinia viruses expressing the N but not G protein. Our data also support and extend the observations of Pemberton et al. (39), who reported that inoculation of mice with recombinant vaccinia viruses expressing the F or N protein induced cytotoxic T cells, whereas inoculation of mice with recombinants expressing the SH or G protein or a portion of the M protein did not. In our studies, we also observed low but significant and reproducible lysis of target cells infected with a recombinant vaccinia virus expressing the P protein. The relatively high recognition of 22K versus low recognition of F or N and minor recognition of P by CTL induced by RS virus infection could be a function of the relative number of CTL epitopes found on each of these structures, a function of the number of CTL precursors for each of these structures, or a combination of the two. Alternatively, the 22K protein may be displayed abundantly on the surface of cells in a processed form.

It was of interest to determine the effect(s) of repeated stimulation of CTL by RS virus in a manner which might be analogous to that encountered by repeated natural reinfection. However, since repeated infection of experimental animals is difficult to achieve, we attempted to create a reinfection scenario by repeated stimulation of murine spleen CTL with live RS virus in vitro. After the first few stimulations, cytolytic activity appeared to be derived exclusively from a $CD8^+$ subset. Continued stimulation generated cultures with mixed phenotype, and cytolytic activity was derived from both a $CD8^+$ and a $CD4^+$ subset. Continued stimulation generated pure cultures of $CD4^+$ cytolytic cells. Although our studies did not directly address the MHC class restriction of our $CD4^+$ CTL, these cells lost cytolytic activity on BCH_4 cells, which express only class I MHC determinants, strongly implicating class II MHC restriction. Our inability to detect RS virus-specific $CD4^+$ CTL in early bulk cultures of mouse spleen suggests that at the precursor level, these effectors are rare after primary infection. However, the sequential exchange of $CD8^+$ CTL with $CD4^+$ CTL occurred in three of three independently derived murine CTL lines and one human CTL line, thus providing evidence for high reproducibility of this event. The outgrowth of $CD4^+$ CTL could be attributable to undefined selection conditions imposed in vitro, or the outgrowth of $CD4^+$ CTL may be contingent upon multiple exposures to RS virus. This latter hypothesis is particularly attractive in light of rapid generation of $CD4^+$ CTL from human PBMC after limited (three to five) stimulations in vitro, since multiple stimulations might be expected to have occurred previously in vivo in an adult donor preselected for high proliferative responsiveness to RS virus.

Although murine $CD4^+$ CTL were potently lytic for RS virus-infected cells bearing class II MHC determinants, none of the three independently derived $CD4^+$ CTL lines recognized Ia^+ cells infected with any of our panel of recombinant vaccinia viruses, including 22K, F, G, SH, M, N, or P. Lack of recognition of cells infected with the recombinant vaccinia viruses did not appear to be a result of lack of expression of appropriate levels of the recombinant protein, especially when considering that others have shown relatively low levels of antigen to be required for recognition by class I- or class II-restricted CTL (24, 41). Thus, it is possible that the target structure(s) for $CD4^+$ CTL is among the two nonstructural 1B or 1C proteins or the polymerase protein, lacking from our tested panel. Alternatively, we cannot dismiss the possibility that the target proteins for RS virus-specific $CD4^+$ CTL are processed differently after endogenous synthesis by vaccinia virus- compared with RS virus-infected cells. This premise is consistent with previous studies showing distinct differences in antigen presentation requirements for class I- and class II-restricted CTL (33, 35) and the demonstration that MHC class II-restricted CTL specific for influenza hemagglutinin do not recognize the hemagglutinin gene product when introduced via a recombinant vaccinia virus vector (35). However, it has been shown that transfected cell lines expressing endogenously produced measles virus proteins may serve as target cells for class II-restricted CTL (24). Whereas it is well known that class II-restricted presentation of antigen to $CD4^+$ T-helper cells requires endosomal processing of (typically) exogenous antigen, little is known of processing requirements for class II-restricted presentation to $CD4^+$ CTL, and we are presently researching these possibilities toward identification of the target structure for our $CD4^+$ CTL.

$CD4^+$ CTL have been defined in several viral systems, including the paramyxovirus measles virus (23, 24, 45), for which class II MHC restriction has been established. It will be important to further characterize $CD4^+$ CTL induction by RS virus, since class II MHC determinants are inducible on a number of cell types accessible to RS virus infection in vivo, including alveolar epithelium, fibroblasts, and endothelial cells (18, 26, 32).

ACKNOWLEDGMENTS

We express our appreciation to Michael Wathen and Ed Hausknecht for providing the purified FG glycoprotein, to Roger Brideau for providing Formalin-inactivated RS virus and for vaccinating mice, and to Robert Olmsted for assistance in constructing some of the recombinant vaccinia viruses. We also thank Martin Sanders for leukapheresis of human donors and Greg Winterrowd for assistance with magnetic bead separation of human lymphocytes. We thank Mary Meissner for dependable and continued excellent secretarial expertise and Robert Chanock for reviewing the manuscript.

ADDENDUM IN PROOF

After the manuscript was submitted, others independently reported that the 22K protein was the major target antigen for *H-2*^{d}-restricted CTL (P. J. M. Openshaw, K. Anderson, G. W. Wertz, and B. A. Askonas, J. Virol. **64**:1683–1689, 1990).

LITERATURE CITED

1. **Bangham, C. R. M., and B. A. Askonas.** 1986. Murine cytotoxic T cells specific to respiratory syncytial virus recognize different antigenic subtypes of the virus. J. Gen. Virol. **67**:623–629.
2. **Bangham, C. R. M., P. J. M. Openshaw, L. A. Ball, A. M. Q. King, G. W. Wertz, and B. A. Askonas.** 1986. Human and murine cytotoxic T cells specific to respiratory syncytial virus recognize the viral nucleoprotein (N), but not the major glycoprotein (G), expressed by vaccinia virus recombinants. J. Immunol. **137**:3973–3977.

3. **Bangham, C. R. M., and A. J. McMichael.** 1986. Specific human cytotoxic T cells recognize B-cell lines persistently infected with respiratory syncytial virus. Proc. Natl. Acad. Sci. USA **83**:9183–9187.
4. **Baybutt, H. N., and C. R. Pringle.** 1987. Molecular cloning and sequencing of the F and 22K membrane protein genes of the RSS-2 strain of respiratory syncytial virus. J. Gen. Virol. **68**:2789–2796.
5. **Brideau, R. J., R. R. Walters, M. A. Stier, and M. W. Wathen.** 1989. Protection of cotton rats against human respiratory syncytial virus by vaccination with a novel chimeric FG glycoprotein. J. Gen Virol. **70**:2637–2644.
6. **Cannon, M. J., P. J. M. 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.
7. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of beta-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5**:3403–3409.
8. **Chanock, R. M., H. W. Kim, C. D. Brandt, and R. H. Parrott.** 1976. Respiratory syncytial virus, p. 365–382. *In* A. S. Evans (ed.), Viral infections of humans. Plenum Publishing Corp., New York.
9. **Chiba, Y., Y. Higashidate, K. Suga, K. Honjo, H. Tsutsumi, and P. L. Ogra.** 1989. Development of cell-mediated cytotoxic immunity to respiratory syncytial virus in human infants following naturally acquired infection. J. Med. Virol. **28**:133–139.
10. **Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette.** 1989. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. **89**:449–463.
11. **Collins, P. L., K. Anderson, S. J. Langer, and G. W. Wertz.** 1985. Correct sequence for the major nucleocapsid protein mRNA of respiratory syncytial virus. Virology **146**:69–77.
12. **Collins, P. L., and G. W. Wertz.** 1985. The envelope-associated 22K protein of human respiratory syncytial virus: nucleotide sequence of the mRNA and a related polytranscript. J. Virol. **54**:65–71.
13. **Elango, N., G. A. Prince, B. R. Murphy, S. Venkatesan, R. M. Chanock, and B. Moss.** 1986. Resistance to human respiratory syncytial virus (RSV) infection induced by immunization of cotton rats with a recombinant vaccinia virus expressing the RSV G glycoprotein. Proc. Natl. Acad. Sci. USA **83**:1906–1910.
14. **Fernald, G. W., J. R. Almond, and F. W. Henderson.** 1983. Cellular and humoral immunity in recurrent respiratory syncytial virus infections. Pediatr. Res. **17**:753–758.
15. **Fernie, B. F., E. C. Ford, and J. L. Gerin.** 1981. The development of Balb/c cells persistently infected with respiratory syncytial virus: presence of ribonucleoprotein on the cell surface. Proc. Soc. Exp. Biol. Med. **167**:83–86.
16. **Fulginiti, A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Miniamitani, 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.
17. **Glezen, W. P., L. H. Taber, A. L. Frank, and J. A. Kasel.** 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am. J. Dis. Child. **140**:543–546.
18. **Halloran, P. F., A. Wadgymar, and P. Autenried.** 1986. The regulation of expression of major histocompatibility complex products. Transplantation **41**:413–420.
19. **Henderson, F. W., A. M. Collier, W. A. Clyde, Jr., and F. W. Denny.** 1979. Respiratory syncytial virus infections, reinfections and immunity. N. Engl. J. Med. **300**:530–534.
20. **Huang, Y. T., P. L. Collins, and G. W. Wertz.** 1985. Characterization of the 10 proteins of human respiratory syncytial virus: identification of a fourth envelope-associated protein. Virus Res. **2**:157–173.
21. **Isaacs, D., C. R. M. Bangham, and A. J. McMichael.** 1987. Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. Lancet **ii**:769–771.
22. **Issekutz, T., E. Chu, and R. S. Geha.** 1982. Antigen presentation by human B cells: T cell proliferation induced by Epstein-Barr virus B lymphoblastoid cells. J. Immunol. **129**:1446–1450.
23. **Jacobson, S., and W. E. Biddison.** 1986. Virus-specific HLA class II-restricted cytotoxic T cells, p. 187–192. *In* M. B. A. Oldstone and A. Notkins (ed.), Concepts in viral pathogenesis II. Springer-Verlag, New York.
24. **Jacobson, S., R. P. Sekaly, C. L. Jacobson, H. F. McFarland, and E. O. Long.** 1989. HLA class II-restricted presentation of cytoplasmic measles virus antigens to cytotoxic T cells. J. Virol. **63**:1756–1762.
25. **Johnson, P. R., and P. L. Collins.** 1989. The 1B(NS2), 1C(NS1) and N proteins of human respiratory syncytial virus (RSV) of antigenic subgroups A and B: sequence conservation and divergence within RSV genomic RNA. J. Gen. Virol. **70**:1539–1547.
26. **Kallenberg, C. G. M., B. M. Schilizzi, F. Beaumont, S. Poppema, L. DeLeij, and T. H. The.** 1987. Expression of class II MHC antigens on alveolar epithelium in fibrosing alveolitis. Clin. Exp. Immunol. **67**:182–190.
27. **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.
28. **Kim, H. W., J. C. 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.
29. **Kim, H. W., S. L. Leikin, J. Arrobio, C. D. Brandt, R. M. Chanock, and R. H. Parrott.** 1976. Cell-mediated immunity to respiratory syncytial virus inducted by inactivated vaccine or by infection. Pediatr. Res. **10**:75–78.
30. **Lopez, J. A., N. Villanueva, J. A. Melero, and A. Portela.** 1988. Nucleotide sequence of the fusion and phosphoprotein genes of human respiratory syncytial (RS) virus Long strain: evidence of subtype genetic heterogeneity. Virus Res. **10**:249–262.
31. **Marchioli, C. C., R. J. Yancey, E. A. Petrovskis, J. G. Timmins, and L. E. Post.** 1987. Evaluation of pseudorabies virus glycoprotein gp50 as a vaccine for Aujesky's disease in mice and swine: expression by vaccinia virus and Chinese hamster ovary cells. J. Virol. **61**:3977–3982.
32. **Maurer, D. H., J. H. Hanke, E. Mickelson, R. R. Rich, and M. S. Pollack.** 1987. Differential presentation of HLA-DR, DQ and DP restriction elements by interferon-gamma-treated dermal fibroblasts. J. Immunol. **139**:715–723.
33. **Moore, M. W., F. R. Carbone, and M. J. Bevan.** 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. Cell **54**:777–785.
34. **Morrison, L. A., V. L. Braciale, and T. J. Braciale.** 1988. Antigen form influences induction and frequency of influenza-specific class I and class II MHC-restricted cytolytic T lymphocytes. J. Immunol. **141**:363–368.
35. **Morrison, L. A., A. E. Lukacher, V. L. Braciale, D. P. Fan, and T. J. Braciale.** 1986. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. J. Exp. Med. **163**:903–921.
36. **Murphy, B. R., A. Stonikov, P. R. Paradiso, S. W. Hildreth, A. B. Jenson, R. B. Baggs, L. Lawrence, J. J. Zubak, R. M. Chanock, J. A. Beeler, and G. A. Prince.** 1989. Immunization of cotton rats with the fusion (F) and large (G) glycoproteins of respiratory syncytial virus (RSV) protects against RSV challenge without potentiating RSV disease. Vaccine **7**:533–540.
37. **Olmsted, R. A., and P. L. Collins.** 1989. The 1A protein of respiratory syncytial virus is an integral membrane protein present as multiple, structurally distinct species. J. Virol. **63**: 2019–2029.
38. **Olmsted, R. A., N. Elango, G. A. Prince, B. R. Murphy, P. R. Johnson, B. Moss, R. M. Chanock, and P. L. Collins.** 1986. Expression of the F glycoprotein of respiratory syncytial virus by a recombinant vaccinia virus: comparison of the individual

contributions of the F and G glycoproteins to host immunity. Proc. Natl. Acad. Sci. USA **83**:7462–7466.

39. **Pemberton, R. M., M. J. Cannon, P. J. M. Openshaw, L. A. Ball, G. W. Wertz, and B. A. Askonas.** 1987. Cytotoxic T cell specificity for respiratory syncytial virus proteins: fusion protein is an important target antigen. J. Gen. Virol. **68**:2177–2182.
40. **Prince, G. A., A. B. Jenson, V. G. Hemming, B. R. Murphy, E. E. Walsh, R. L. Horswood, and R. M. Chanock.** 1986. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of Formalin-inactivated virus. J. Virol. **57**:721–728.
41. **Puddington, L., M. J. Bevan, J. K. Rose, and L. LeFrancois.** 1986. N protein is the predominant antigen recognized by vesicular stomatitis virus-specific cytotoxic T cells. J. Virol. **60**:708–717.
42. **Satake, M., N. Elango, and S. Venkatesan.** 1984. Sequence analysis of the respiratory syncytial virus phosphoprotein gene. J. Virol. **52**:991–994.
43. **Satake, M., and S. Venkatesan.** 1984. Nucleotide sequence of the gene encoding respiratory syncytial virus matrix protein. J. Virol. **50**:92–99.
44. **Taylor, G., E. J. Stott, and A. J. Hayle.** 1985. Cytotoxic lymphocytes in the lungs of mice infected with respiratory syncytial virus. J. Gen. Virol. **66**:2533–2538.
45. **Van Binnendijk, R. S., M. C. M. Poelen, P. DeVries, P. H. O. Voorma, A. D. M. E. Osterhaus, and F. G. C. M. Uytdehaag.** 1989. Measles virus-specific human T cell clones: characterization of specificity and function of $CD4^+$ helper/cytotoxic and $CD8^+$ cytotoxic T cell clones. J. Immunol. **142**:2847–2854.
46. **Wathen, M. W., R. J. Brideau, D. R. Thomsen, and B. R. Murphy.** 1989. Characterization of a novel human respiratory syncytial virus chimeric FG glycoprotein expressed using a baculovirus vector. J. Gen. Virol. **70**:2625–2635.
47. **Wolcott Nicholas, J. A., M. E. Levely, R. J. Brideau, and A. E. Berger.** 1987. During recovery from cytomegalovirus infection T-lymphocyte subsets become selectively responsive to activation and have depressed interleukin 2 (IL2) secretion and IL2 receptor expression. Microb. Pathog. **2**:37–47.