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The Reovirus Nonstructural Protein σ1NS Is Recognized by Murine Cytotoxic T Lymphocytes

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The cytotoxic T-lymphocyte (CTL) response in reovirus-infected C3H mice was investigated by using reovirus-vaccinia virus recombinants. Results of cytotoxicity assays indicated that the nonstructural protein σ1NS elicited a significant CTL response. Experiments with σ1NS-specific CTL lines showed that both strain-specific and cross-reactive epitopes exist in the σ1NS protein.

The mammalian reoviruses are nonenveloped viruses with an icosahedral, double-layered capsid shell and a genome consisting of 10 segments of double-stranded RNA. The 10 genomic segments code for 11 proteins, with the S1 gene segment encoding a bicistronic mRNA that is translated into two proteins, σ1 and σ1NS (5, 11, 17). The σ1 protein is responsible for attachment of reoviruses to cells (13) and determines much of the tropism and patterns of pathogenesis during reovirus infection in animals and cell culture (20). σ1 also determines the serologic type specificity among the three serotypes of reovirus (18). σ1NS is a nonstructural protein of unknown function.

Reovirus has been studied extensively in the mouse as a model system for investigating virus-induced pathogenesis. The cytotoxic T-lymphocyte (CTL) response to reovirus in the mouse was initially characterized by Finberg et al. (9), who showed that (i) cytotoxic effector cells were classic CTL by their $Thy1^{+}$ phenotype and *H-2*-restricted reactivity; (ii) the cytotoxic response to reovirus infection was serotype specific, with maximal cytolytic activity observed with target cells infected with the serotype used to induce the CTL; and (iii) the S1 gene segment encoded a protein with which CTL react. These experiments were conducted by using reassortant reoviruses at a time when σ1NS was not yet discovered, and the CTL response observed was assumed to be against the σ1 protein. Additional studies by Finberg et al. (7, 8) demonstrated that serotype-specific antiserum blocked CTL-target cell recognition and that the neutralization domain of σ1 was the dominant CTL-reactive epitope on the basis of studies with monoclonal antibodies to σ1. Subsequently, London et al. (15) and Parker and Sears (16) reached a conclusion opposite that of Finberg et al. (7–9), namely, that reovirus-specific CTL are not reovirus type specific, since the CTL did not preferentially lyse targets infected with the homologous serotype of reovirus.

The specificity of reovirus-induced CTL was further defined by Hogan and Cashdollar (10) by clonal analysis of CTL. This analysis revealed two different types of immune recognition: (i) a serotype-specific response demonstrated by a single clone exhibiting specificity for only type 3 reovirus-infected targets, with no reactivity for type 1- or 2-infected targets, and (ii) a cross-reactive response demonstrated by three clones that exhibited cross-reactivity between type 1- and 3-infected cells, with low levels of cross-reactivity against type 2-infected cells.

The present study utilized vaccinia virus-reovirus recombinants to examine the CTL response to the proteins encoded by the S1 genome segment during reovirus infection. We report that CTL react to the reovirus nonstructural protein, σ1NS, and that both strain-specific and cross-reactive CTL exist.

Establishment of CTL lines. C3H ($H\text{-}2^k$) mice were obtained from Jackson Laboratories (Bar Harbor, Maine) at 4 to 6 weeks of age. The mice were immunized at 6 to 12 weeks of age. Animals were maintained under germ-free conditions in the Biocontainment Suite of the Animal Resource Center at the Medical College of Wisconsin. CTL were obtained from either the lymph nodes or spleens after mice were immunized by one of two different routes. Five days after the hind footpads of mice were inoculated with 10^7 PFU of reovirus type 3 (strain Dearing) in 25 μl of sterile phosphate-buffered saline (PBS), mice were sacrificed, and the draining popliteal lymph nodes were harvested. Alternatively, 7 days after intraperitoneal inoculation with 10^7 PFU of reovirus type 3 in 250 μl of sterile PBS, mice were sacrificed and the spleens were harvested as described previously (10).

CTL cultures were obtained from either lymph nodes or spleens by selection of cells that grew in response to in vitro stimulation with reovirus-infected stimulator cells. A single-cell suspension of effector cells was made from each pair of draining popliteal lymph nodes. The lymph node cells were cultured in 24-well tissue-culture plates at a concentration of 1.75×10^6 cells per well in the presence of 1.2×10^5 to 2.5×10^6 syngeneic feeder cells and 5×10^5 stimulator cells. The feeder cells were cesium-irradiated (3,000 rads) splenocytes, and the stimulator cells were irradiated (10,000 rads) Ltk− or L929 cells ($H\text{-}2^k$) infected with reovirus serotype 3 at 5 PFU per cell.

A single-cell suspension of effector cells was made from each spleen from mice inoculated through intraperitoneal injection. Effector cells were cultured in 24-well plates at a concentration of 5×10^6 cells per well in the presence of 5×10^5 stimulator cells.

CTL lines derived from lymph nodes or spleens were restimulated weekly by culturing 7×10^5 effector cells with 5×10^5 stimulator cells and 1.2×10^5 to 2.5×10^6 syngeneic feeder cells per well of a 24-well plate or 1.5×10^6 effector cells with 1×10^6 stimulator cells and 3×10^6 syngeneic feeder cells per well of a 6-well plate.

CTL lines were stimulated in vitro at least once but more often two or three times before being used in cytotoxicity

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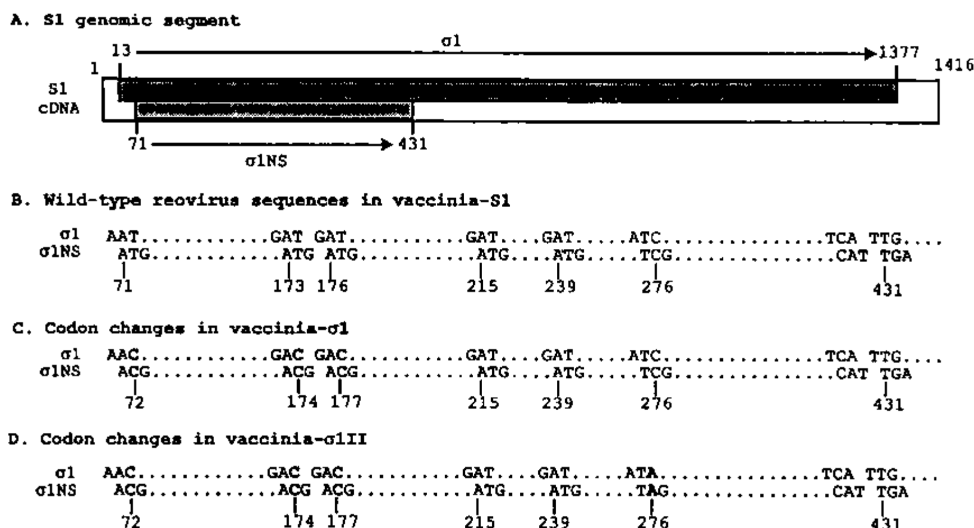

FIG. 1. Reovirus coding sequences in the S1 genomic segment (A) and the vaccinia virus recombinants (B to D). Numbers indicate base pairs in the S1 cDNA sequence.

assays. The σ1NS-specific CTL lines were cultured for several weeks before being used in cytotoxicity assays.

All CTL lines were cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS) and 10 U of recombinant interleukin-2 per ml at 37°C. The medium was supplemented with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1 mM pyruvic acid, 2 mM L-glutamine, essential and nonessential amino acids, vitamins, 35 μg of gentamicin per ml, and 5×10^{-5} M β-mercaptoethanol. CTL cultures were periodically layered onto a cushion of lymphocyte separation medium (Organon Teknika Corporation) and centrifuged to separate viable CTL from cell debris and dead cells.

Cytotoxicity assays. To measure cell lysis by CTL, virus-infected L cells were used as targets in chromium release assays. L cells were infected at 5 PFU per cell with reovirus serotypes 1 (strain Lang) or 3 (strain Dearing) or with reassortant virus 3HA1 (a monoreassortant with the S1 genome segment from serotype 1 and the remaining segments from serotype 3) or 1HA3 (a monoreassortant with the S1 genome segment from serotype 3 and the remaining segments from serotype 1) (22). Reassortants 3HA1 and 1HA3 were obtained from Max Nibert, University of Wisconsin, Madison. Vaccinia virus recombinants expressing various reovirus proteins were used to infect targets at 1 to 3 PFU per cell. These multiplicities of infection were empirically determined to give the best presentation of antigen with the least amount of cytotoxicity due to the virus infection. Immunofluorescence microscopy of infected target cells showed that greater than 95% were infected with reovirus at 5 PFU per cell and 60 to 95% were infected with vaccinia virus recombinants at 1 to 3 PFU per cell, respectively. These infection rates are consistent with the theoretical values calculated using the Poisson distribution. Both reovirus and vaccinia virus recombinant targets were incubated for 12 h at 37°C. Both infected and uninfected targets were harvested by scraping them into medium, and they were then incubated with 100 to 200 μCi of $Na_2{}^{51}Cr_2O_7$ (New England Nuclear; 1,000 μCi/ml) for 2 h. The cells were washed three times with RPMI 1640 supplemented with 1% FBS, a viable cell count was obtained, and the cells were resuspended in RPMI 1640 with 5% FBS for use in CTL assays.

^{51}Cr release assays were performed in triplicate with four dilutions of effector cells plated with 5×10^3 ^{51}Cr-labeled target cells per well in a 96-well microtiter plate. The cells were incubated at 37°C for 4 h. The percent specific release (%SR) was calculated as $(ER - SPR)/(MR - SPR) \times 100$, where ER is the experimental release, SPR is the spontaneous release obtained in the absence of effector cells, and MR is the maximum release obtained in the presence of 2% Triton X-100. The spontaneous chromium release ranged from 5 to 20%, with the majority of the targets showing SPR values of 10% or less. The standard deviations of the triplicate determinations were routinely less than 5%.

Construction of vaccinia virus-reovirus recombinants. The cDNA sequences of reovirus genes were excised from various plasmids and subcloned into the vaccinia virus expression plasmid pSC11 (4) (obtained from Bernard Moss, National Institutes of Health). The reovirus serotype 3 S4 cDNA was derived from the original clone in pBR322 (3). The cDNA was excised from pBR322 with *Tth*111I which cleaves at nucleotide 25 in the 5' untranslated region of the S4 cDNA and with *Pst*I at the 3' end. The ends of the cDNA fragment were made blunt, and it was cloned into the *Sma*I site of pSC11.

The coding sequence for the serotype 3 σ1NS protein was excised from plasmid pG4T3 (14) by digestion with *Bam*HI and *Nco*I. This fragment included nucleotides 15 to 566 of the S1 cDNA which did not include the initiation codon for the σ1 reading frame. The ends of the fragment were made blunt with Klenow, and the fragment was cloned into the *Sma*I site of pSC11.

The constructs that were designed to produce σ1 either in the presence or absence of σ1NS are illustrated in Fig. 1. The complete coding region for σ1 was present in each construction, with the indicated mutations designed to eliminate translation from the σ1NS reading frame without changing the amino acid composition of the σ1 protein. The mutations made in vaccinia virus-σ1 were those made in the plasmid pCos-Mtn$_2$ (6), and the mutations made in vaccinia virus-σ1II were those made in pS-SDMT3. The addition of an in-frame stop codon in the σ1II construct eliminated the possibility of a truncated portion of σ1NS being synthesized by this recombinant via initiation from codons at bp 215 or 239. Plasmids pG4T3, pCos-Mtn$_2$, and pS-SDMT3 were obtained from Patrick W. K. Lee, Calgary, Alberta, Canada. The σ1 coding

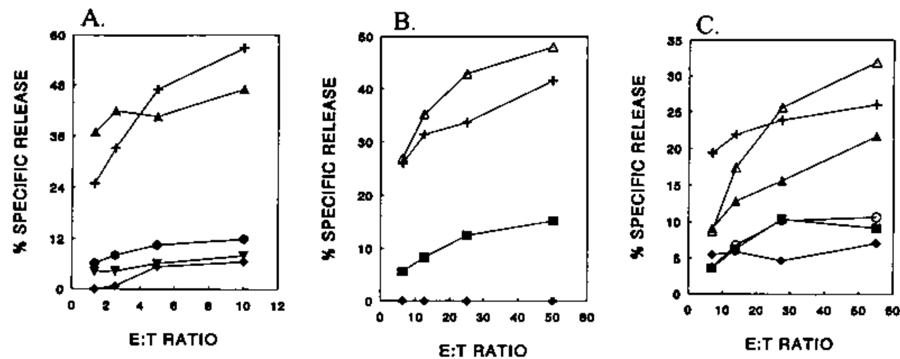

FIG. 2. ^{51}Cr release cytotoxicity assays of reovirus-specific CTL. The CTL lines used as effector cells in panels A and B were derived from the splenocytes of reovirus-infected mice. The CTL line used as effector cells in panel C was derived from lymph node cells from reovirus-infected mice. Results are expressed as the percent specific release of each target at the indicated effector-to-target (E:T) cell ratios. Each point is the average of triplicate determinations. Targets: ◆, uninfected; +, reovirus; ▼, vaccinia virus; ▲, S1 recombinant; ●, S4 recombinant; ■, σ1 recombinant; ○, σ1II recombinant; △, σ1NS recombinant.

sequences shown in Fig. 1 were cloned into the *Sma*I site of pSC11.

The reovirus-specific sequences that had been cloned into pSC11 were inserted into the vaccinia virus (strain WR, obtained from Bernard Moss) genome by the standard methods of homologous recombination (23). Vaccinia recombinant viruses were propagated in mouse L cells. Plaque assays of wild-type and recombinant vaccinia virus stocks were performed on human 143 B TK− cells (American Type Culture Collection).

CTL reactivity to reovirus σ1NS protein. The response of splenic- and lymph node-derived CTL cultures to specific reovirus proteins was analyzed using target cells infected with vaccinia virus-reovirus recombinants in ^{51}Cr release assays (Fig. 2). Splenocyte cultures were prepared from C3H mice that were immunized with reovirus serotype 3 and stimulated in vitro with reovirus as described above. The CTL line in panel A of Fig. 2 showed reactivity to reovirus-infected targets which indicated the expected reovirus specificity. This CTL line also showed reactivity to the vaccinia virus-reovirus S1 gene recombinant, whereas the vaccinia virus-reovirus S4 gene recombinant gave specific release values that were barely above the background values of the cell control and vaccinia virus control targets.

Since the S1 gene segment encodes a bicistronic mRNA for the σ1 and σ1NS proteins, it was necessary to separate these two reading frames using recombinant constructions in vaccinia virus to determine the identity of the reactive protein. Vaccinia virus-reovirus recombinants that expressed only the σ1NS or σ1 protein were constructed as described above. Targets infected with the recombinant virus expressing the σ1NS protein were recognized by the CTL, while targets infected with the vaccinia virus-reovirus σ1 construct showed specific release that was only slightly above the background values of both the uninfected and vaccinia virus-infected targets, whose curves are superimposible (Fig. 2B).

CTL from lymph node cultures were also analyzed for a response to the σ1 and σ1NS proteins. Recognition of viral proteins by CTL from the lymph node cultures was comparable to that obtained with CTL from the splenic cultures. As observed with splenocyte-derived CTL lines, targets infected with vaccinia virus-reovirus S1 or σ1NS were good targets for lymph node-derived CTL lines, while targets infected with vaccinia virus-reovirus σ1 were not (Fig. 2C). To verify the lack of a response to the σ1 protein, the σ1II recombinant was evaluated in this assay with the lymph-node CTL cultures. Both the σ1 and σ1II recombinants were minimally recognized by the CTL lines. For the remainder of the experiments reported here, lymph node-derived CTL cultures were used because of their ease of establishment and the high percentages of CD8-positive cells (>90%) that were obtained with successive in vitro stimulations.

To ensure that all of the vaccinia virus-reovirus recombinants that were designed to synthesize σ1 were doing so, L929 cells infected with each of the σ1-encoding constructs were tested by Western immunoblot for the production of σ1. The results indicated that σ1 was synthesized during infection of L cells with the S1, σ1, and σ1II constructs and therefore a lack

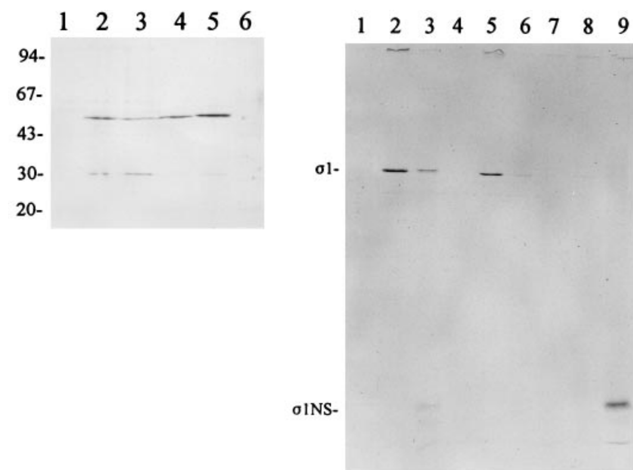

FIG. 3. (Left) Immunoblot analysis of σ1 protein produced by reovirus-vaccinia virus recombinants. L929 cells were infected with reovirus at 5 PFU per cell and at 3 PFU per cell with vaccinia virus and each of the recombinant viruses. Sixteen hours after infection the cells were lysed in sodium dodecyl sulfate-gel loading buffer. Lysate equivalent to 10^5 cells was loaded into each lane of the gel. The blot was probed with polyclonal anti-σ1 antibody, and the antigen-antibody complexes were detected with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G with the enhanced chemiluminescence detection system (Amersham). Lanes: 1, vaccinia virus strain WR; 2, S1 recombinant; 3, σ1 recombinant; 4, σ1II recombinant; 5, reovirus serotype 3; 6, σ1NS recombinant. (Right) Immunoprecipitation of [^{35}S]methionine-labeled infected cell lysates. Cells were infected as described above and labeled with [^{35}S]methionine (100 μCi per ml) at 12 h postinfection. Cells were harvested at 16 h postinfection, and cell lysates were immunoprecipitated with anti-σ1 or anti-σ1NS specific antibody. Lanes: 1 to 3, infection with the S1 recombinant; 4 to 6, infection with the σ1 recombinant; 7 to 9, infection with the σ1NS recombinant. Lanes 1, 4, and 7 contain normal rabbit serum; lanes 2, 5, and 8 contain polyclonal anti-σ1 antibody; and lanes 3, 6, and 9 contain polyclonal anti-σ1NS antibody.

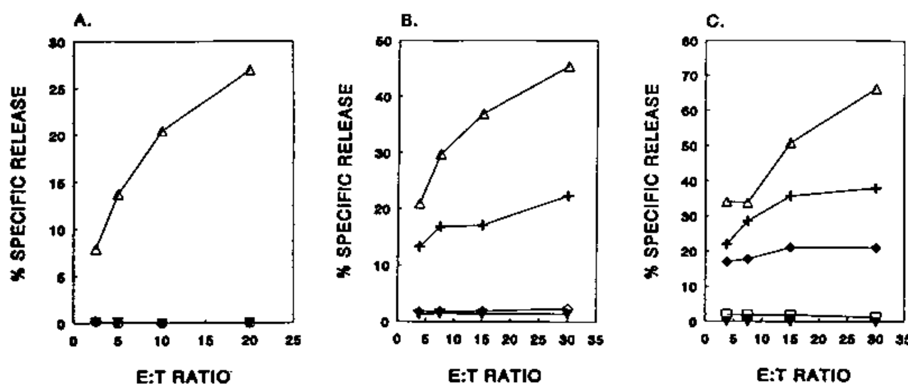

FIG. 4. [51]Cr release cytotoxicity assays of reovirus type 3 σ1NS-specific CTL line A. (A) σ1NS specificity of CTL line A. (B and C) Serotype-specific reactivity of CTL line A. Results are expressed as percent specific release as described in the legend to Fig. 2. Targets: ◇, reovirus type 1; +, reovirus type 3; ◆, reovirus 1HA3; □, reovirus 3HA1; ○, σ1II recombinant; △, σ1NS recombinant; ▼, vaccinia virus strain WR.

of σ1 protein production could not explain the difference in CTL reactivity between the S1 construct and the two σ1 constructs (Fig. 3, left panel). The bands of lower molecular weight that appear in the Western blot are probably degradation products of σ1 which appear in various amounts with different lysate preparations.

The synthesis of σ1NS in the S1 and σ1NS recombinants was confirmed by immunoprecipitation assays using σ1NS-specific antibody that we prepared using a bacterial fusion protein as an antigen (2) (Fig. 3, right panel). As expected, both σ1 and σ1NS are synthesized with the S1 recombinant, whereas the σ1 and σ1NS recombinants produced only σ1 and σ1NS proteins, respectively. The antibody used to immunoprecipitate σ1NS also precipitates σ1 with lesser efficiency, as is illustrated in lane 3. The amount of σ1NS produced with the S1 construct is less than that produced from equivalent infection with the σ1NS recombinant, probably due to initiation of translation from the σ1 reading frame.

Strain specificity of the σ1NS CTL response. In order to investigate the strain specificity of the CTL response to the σ1NS protein, CTL specific for the reovirus type 3 σ1NS protein were generated by first inoculating mice with reovirus type 3 by footpad injection. The draining lymph nodes from two mice were harvested 5 days after injection and restimulated in vitro with virus-infected L cells. Two methods of restimulation were utilized to generate CTL lines from mice A and B. CTL line A was restimulated in vitro only with the σ1NS vaccinia recombinant virus. CTL line B was restimulated in vitro once with reovirus type 3-infected L cells and thereafter with σ1NS vaccinia recombinant virus. Both cell lines experienced a period of crisis early during culture as the number of viable cells detected by trypan blue dye exclusion decreased for several weeks. Eventually the CTL lines stabilized and the number of viable cells began to gradually increase.

Since the CTL lines were stimulated in vitro with σ1NS vaccinia recombinant virus, it was expected that these CTL would react specifically with that protein. The cytotoxicity data with line A confirmed the reactivity to σ1NS and the lack of reactivity to vaccinia virus-infected targets as well as targets expressing σ1 (Fig. 4A). The σ1NS-specific CTL line A was then used to test the strain specificity of the reactivity. Reovirus serotypes 1 (Lang) and 3 (Dearing) were used to prepare targets for CTL as well as σ1NS and vaccinia virus as positive and negative controls, respectively. CTL reactivity to σ1NS was specific for serotype 3 (strain Dearing) σ1NS (Fig. 4B). Confirmation of the strain specificity of σ1NS CTL was obtained by using reassortant viruses 3HA1 and 1HA3 to create targets for cytotoxicity assays. Only target cells producing σ1NS from serotype 3 (Dearing) reovirus were recognized by this CTL line (Fig. 4C). 3HA1 that has a type 1 S1 gene segment was not able to be recognized in this assay.

A different response was seen with σ1NS-specific cell line B which showed a significant degree of cross-reactivity between serotype 1 and 3 reovirus (Fig. 5A). The cross-reactivity to serotype 1 and 3 σ1NS proteins was confirmed by using 3HA1 and 1HA3 reassortant viruses (Fig. 5B).

Using vaccinia virus-reovirus recombinants we have determined that a portion of the CTL isolated in response to reovirus infection of H-2^k mice is specific for the σ1NS protein. Since σ1NS is encoded by the S1 genome segment these data support the original findings of Finberg et al., who mapped the predominant CTL-reactive protein to the S1 gene segment using reassortant viruses (9). In contrast to what would have been predicted from the results of Finberg et al., we detected little if any reactivity to the σ1 protein. These data suggest that much of the CTL reactivity seen by Finberg et al. could have been specific for the σ1NS protein. Our data are not consistent with other results reported by Finberg et al., who reported that

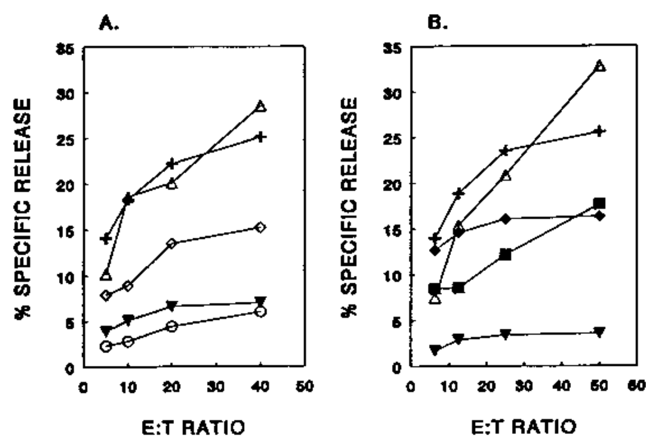

FIG. 5. [51]Cr release cytotoxicity assays of reovirus type 3 σ1NS-specific CTL line B. (A) Serotype cross-reactivity between reovirus types 1 and 3. (B) Serotype cross-reactivity demonstrated with reovirus reassortants. Results are expressed as percent specific release as described in the legend to Fig. 2. Targets: ◇, reovirus type 1; +, reovirus type 3; ◆, reovirus 1HA3; ■, reovirus 3HA1; ○, σ1II recombinant; △, σ1NS recombinant; ▼, vaccinia virus strain WR.

type-specific antibody could block CTL reactivity and that the CTL-reactive epitope on σ1 was part of the neutralization domain defined by σ1-specific monoclonal antibodies (7, 8). These results are difficult to reconcile with our present understanding of the processing and presentation of virus-derived epitopes. It is possible that the slight CTL response we detected to σ1 in the experiments presented here was related to the σ1 reactivity seen in the experiments reported by Finberg et al. To address this possibility, experiments are in progress to attempt to isolate σ1-specific CTL lines using methods similar to those presented here for the isolation of σ1NS-specific CTL lines.

Although Finberg et al. concluded that the CTL response was serotype specific, the findings of London et al. (15) and Parker and Sears (16) indicated that the CTL response was cross-reactive. Our results with CTL lines that were type 3 (strain Dearing) σ1NS-specific showed both strain-specific and cross-reactive responses. These data suggest that there are two CTL-reactive epitopes in the σ1NS protein. It is possible that the slight difference in the in vitro stimulation protocol between the two lines (line B having its first in vitro stimulation with reovirus rather than vaccinia virus-σ1NS) could have preferentially selected one line over the other. Both lines underwent a crisis period during which the viable cell count dropped; presumably it was during this period that the σ1NS-specific CTL were selected and any other reovirus-specific CTL died from a lack of antigenic stimulation. At this point we do not know whether the type-specific or the cross-reactive response is predominant. This question will be addressed in future experiments using precursor frequency analysis. Precursor frequency data will also provide an indication of the predominance of the CTL response to σ1NS in the context of the overall reovirus-specific CTL response. We do not know if the cross-reactive response reported by London et al. and Parker and Sears is the same cross-reactive σ1NS response we observed or if another reovirus protein is responsible for eliciting this response. Other than σ1 and σ1NS, the only reovirus protein tested for its CTL-reactivity in the present study was σ3, which showed little if any reactivity. Data presented here with σ1NS-specific CTL are consistent with our previous findings (10) in which it was demonstrated that a minimum of four epitopes are recognized by reovirus-specific CTL, including both cross-reactive and strain-specific epitopes. Future studies will attempt to correlate our clonal and precursor frequency data (10) with the σ1NS-specific CTL response data presented here.

It is interesting that σ1NS, a reovirus nonstructural protein, elicits a CTL response. σ1NS should now be added to the list of nonstructural virus proteins that are recognized by CTL, which includes the NS1 protein of influenza virus (12), the E1A protein of adenovirus (21), and the ICP27 protein of herpes simplex virus (1). Future experiments will address the importance of the CTL response to σ1NS with regard to the protective immune response to reovirus infection in the mouse.

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