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Identification of an Immunodominant Cytotoxic T-Lymphocyte Recognition Site in Glycoprotein B of Herpes Simplex Virus by Using Recombinant Adenovirus Vectors and Synthetic Peptides

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Cytotoxic T-lymphocyte (CTL) responses to herpes simplex virus (HSV) polypeptides play an important role in recovery from infection and in preventing latency. We have previously shown that glycoprotein B (gB) is a major target recognized by HSV-specific CTLs in C57BL/6 (*H-2^b*) and BALB/c (*H-2^d*) mice but not in CBA/J (*H-2^k*) mice (L. A. Witmer, K. L. Rosenthal, F. L. Graham, H. M. Friedman, A. Yee, and D. C. Johnson, J. Gen. Virol. 71:387–396, 1990). In this report, we utilize adenovirus vectors expressing gB with various deletions to localize an immunodominant site in gB, recognized by *H-2^b*-restricted anti-HSV CTLs, to a region between residues 462 and 594. Overlapping peptides spanning this region were synthesized and used to further localize the immunodominant site to residues 489 to 515, a region highly conserved in HSV type 1 (HSV-1) and HSV-2 strains. The 11-amino-acid peptide was apparently associated exclusively with the *K^b* major histocompatibility complex gene product and not the *D^b* gene product. In contrast, *H-2^d*-restricted CTLs recognized an immunodominant site between residues 233 and 379.

Herpes simplex virus (HSV)-specific cytotoxic T lymphocytes (CTL) play a central role in controlling primary and secondary virus infections, in recovery from infection (for reviews, see references 34 and 41), and in restricting HSV spread into the nervous system (6, 40). CTL recognize small peptides derived from viral proteins which are associated with class I or class II major histocompatibility complex (MHC) polypeptides on the surfaces of virus-infected cells (3, 18, 57, 62). In many cases, CTL recognize a limited number of immunodominant CTL sites in viral polypeptides (for a review, see reference 2). Studies by Townsend et al. (55, 57) demonstrated that synthetic peptide fragments from distinct sites in the nucleocapsid protein of influenza virus were recognized by anti-influenza virus CTL generated in distinct murine strains. Similarly, it was found that the majority of murine *H-2^b*-restricted CTL clones capable of recognizing the lymphocytic choriomeningitis virus glycoprotein were specific for a nine-residue region of the molecule (42, 59), whereas virtually all *H-2^d*-restricted CTL recognized a single site in the lymphocytic choriomeningitis virus nucleoprotein (52, 60). Furthermore, a single 19-residue domain of murine cytomegalovirus immediate-early (IE) protein pp89 was recognized by CTL in BALB/c mice (16). The subset of peptides which become immunodominant for CTL may depend upon the affinity of peptide for MHC molecules (1, 9), as well as T-cell repertoires in the responding strain.

Not only are there strong preferences for specific peptide fragments derived from a single viral polypeptide, but CTL responses to individual viral polypeptides often predominate over responses to other viral proteins (26, 56, 59, 60, 63). In the case of influenza virus and lymphocytic choriomeningitis virus, relatively few viral gene products are expressed in virus-infected cells and thus preferences for individual viral proteins may not be surprising. In contrast, herpesviruses express a large number of viral polypeptides which are regulated in a cascade fashion, with IE proteins being expressed before early and late gene products. Murine cytomegalovirus-specific CTL predominantly recognized the viral IE pp89 polypeptide (47), an observation which may be explained by the fact that viral early or late gene products can act to prevent recognition of murine cytomegalovirus antigens (15) or that a limited number of IE gene products are exclusively expressed for relatively long periods following murine cytomegalovirus infection (for a review, see reference 54). Similarly, human CTL responses to Epstein-Barr virus (EBV) latent antigens predominate over responses to viral proteins expressed during the lytic phase of virus replication (8, 39). This may be explained by the fact that a large majority of EBV-infected B cells exclusively express latent phase proteins (for a review, see reference 31).

HSV also encodes a large number of gene products. However, in contrast to cytomegalovirus and EBV, shifts from one class of viral genes to the next occur more rapidly in HSV-infected cells and most viral gene products are expressed for a large fraction of the infectious cycle. Early gene products, including many structural proteins, can be detected by 2 or 3 h postinfection and late gene products can be detected by 3 or 4 h. Therefore, since HSV early and late polypeptides, as well as IE proteins, are expressed throughout most of the infectious cycle of the virus, CTL preferences for IE proteins may be less likely than with cytomegalovirus and EBV, where IE or latent genes are expressed for long periods.

Previous studies in which drugs were used to block synthesis of early and late gene products suggested that HSV IE gene products can be recognized as CTL targets (32). More recent studies involving virus vectors demonstrated that IE protein ICP4, but not IE protein ICP0, was recognized by *H-2^k* but not *H-2^b* and *H-2^d*-restricted CTL (35). Furthermore, we and others have found that a limited subset

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of HSV glycoproteins, which are early or late gene products, can act as CTL target antigens (4, 5, 19, 30a, 33, 38, 61). Our recent results suggest that glycoprotein B (gB) is a major CTL target antigen in some but not all haplotypes (61). Cells infected with an adenovirus (Ad) vector expressing gB were lysed as efficiently as HSV-infected targets by primary $H\text{-}2^b$-restricted, anti-HSV CTL. Further, gB-expressing cells were recognized in the $H\text{-}2^d$ haplotype but not in the $H\text{-}2^k$ haplotype. By using limiting dilution analysis, we estimated that a minimum of 5 to 10% of the $H\text{-}2^b$-restricted CTL from HSV-infected animals were able to recognize gB (61). Given that HSV expresses upwards of 70 proteins, 8 or more of them glycoproteins, the observation that as many as 10% of the anti-HSV CTL recognized gB was striking. In this report, we have used recombinant Ad vectors and synthetic peptides to define a major CTL site in gB recognized by $H\text{-}2^b$-restricted CTL. Studies involving the Ad vectors suggested that a second immunodominant site in gB is recognized by $H\text{-}2^d$-restricted CTL.

TABLE 1. Restriction fine specificity of $H\text{-}2^b$ CTL recognizing HSV-1 gB

Target cell	Infected with[a]:	% Specific lysis at effector/target cell ratio[b]:		
		40:1	12:1	4:1
MC57G ($H\text{-}2K^bD^b$)	HSV-1	54	33	22
	AdE3$^-$	0	1	4
	AdgB2	28	26	22
HTG ($H\text{-}2K^dD^b$)	HSV	0	2	7
	AdE3$^-$	1	2	8
	AdgB2	0	0	4
5R ($H\text{-}2K^bD^d$)	HSV	28	20	3
	AdE3$^-$	10	10	3
	AdgB2	62	61	9

[a] Target cells were infected with HSV-1, AdE3$^-$ (an E3$^-$ Ad vector which does not express HSV proteins), or AdgB2 and labeled with ^{51}Cr.

[b] Radiolabeled target cells were incubated for 6 h with primary nonadherent lymphocytes from HSV-infected C57BL/6 mice by using various effector-target cell ratios, and the percent specific ^{51}Cr release was calculated.

MATERIALS AND METHODS

Cells and viruses. MC57G ($H\text{-}2^b$) (64), SVBALB ($H\text{-}2^d$) (20), Lta ($H\text{-}2^k$) (21), 5R ($H\text{-}2K^bD^d$) (50), and HTG ($H\text{-}2K^dD^b$) (50) cells were grown in alpha minimal essential medium (α-MEM) (GIBCO Laboratories, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 1% penicillin–streptomycin (GIBCO), and 0.3% L-glutamine. R970-5 cells (48), obtained from K. Huebner and C. Croce (Wistar Institute, Philadelphia, Pa.), and Vero cells were grown in α-MEM supplemented with 7% FBS and penicillin-streptomycin. Ad5-transformed human embryo kidney cells, 293 cells (23), were grown in MEM supplemented with 10% newborn calf serum and penicillin-streptomycin. HSV type 1 (HSV-1) strain F was propagated and virus titers were determined on Vero cells. AdE3$^-$, originally denoted as *dl*E3 (25), AdgB2 (29), and other Ad vectors were grown in KB cell Spinner cultures, and titers of the virus were determined with 293 cell monolayers.

Synthetic peptides. Peptides were synthesized by using an Applied Biosystems peptide synthesizer (Vetrogen Corporation, London, Ontario, Canada). Peptides were 65 to 80% pure for initial screening experiments, and peptides 2 (residues 470 to 481), 5 (489 to 504), 6 (499 to 515), and 5/6 (497 to 507) were purified to >95% purity by using high-pressure liquid chromatography for subsequent experiments. An additional glycine residue was added to the C terminus of peptide 2 to facilitate synthesis and to increase solubility. In all cases, numbering of amino acid residues of gB begins at the N-terminal methionine and includes the signal sequence which is removed from the mature protein. Peptide sequences were predicted by using the HSV-1 KOS sequence (11), but it should be noted that the sequence around the CTL site identified here is identical in HSV strains F and KOS (11, 44). Peptides were dissolved at 10^{-2} M in dimethyl sulfoxide (DMSO) and further diluted at least 100-fold in RPMI 1640 medium (GIBCO) for ^{51}Cr release assays. Peptides 5/6, 8 (residues 521 to 538), and 12 (559 to 575) were not completely soluble in aqueous media at 10^{-4} M, and peptides 8 and 12 showed cytotoxic effects towards target cells in some experiments in which ^{51}Cr was released in the absence of effectors.

Construction of plasmids. All enzymes used for recombinant DNA work were purchased from Bethesda Research Laboratories (Burlington, Ontario, Canada) or Boehringer-Mannheim, Inc. (Dorval, Quebec, Canada) and used under reaction conditions recommended by the vendors. Plasmids were constructed by standard protocols (31a) with *Escherichia coli* LE293. Plasmid DNA was prepared by the alkaline lysis method of Birnboim and Doly (2a). For transfection of 293 cells, plasmid DNA was further purified on CsCl-ethidium bromide density gradients.

Construction of recombinant Ads. Plasmids containing mutated forms of the HSV-1 KOS gB gene were previously described (12). *Hin*dIII-*Bam*HI fragments from pKd1B (in this paper, "d" replaces "Δ" used previously), pKd2, or pKd3 containing mutant gB genes or *Hin*dIII-*Bgl*II fragments from pK157B, pK177B, or pK147B were inserted between the simian virus 40 (SV40) early promoter element and polyadenylation site in pSV2X3 (46). The hybrid SV40-HSV genes were inserted into PFG144XS, which contains the rightward 30 map units of Ad5 DNA with a deletion in the E3 region (20a), so that the insertions were in the left-to-right orientation relative to the conventional Ad5

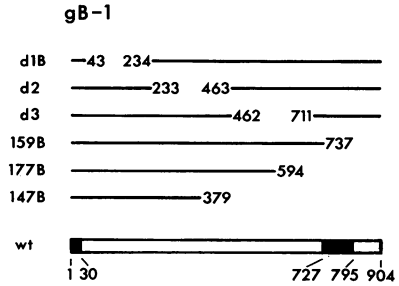


FIG. 1. Structures of deletion mutants of the HSV-1 gB gene. The wild-type (wt) HSV-1 gB contains an N-terminal signal sequence (residues 1 to 30) that is removed during or shortly after synthesis of the protein (13) and a putative transmembrane-spanning domain which may cross the membrane three times (residues 727 to 795) (44). These two regions are indicated by solid bars in the map of gB shown at the bottom. The endpoints of the deletion mutations are also indicated. Mutants d1B, d2, and d3 were constructed by deleting sequences between *Hpa*I restriction sites, and mutants 159B, 177B, and 147B were truncated by removing sequences 3′ to the inserted *Bgl*II sites (12). Numbering of amino acid residues begins at the N-terminal methionine rather than at the alanine of the mature form of gB.

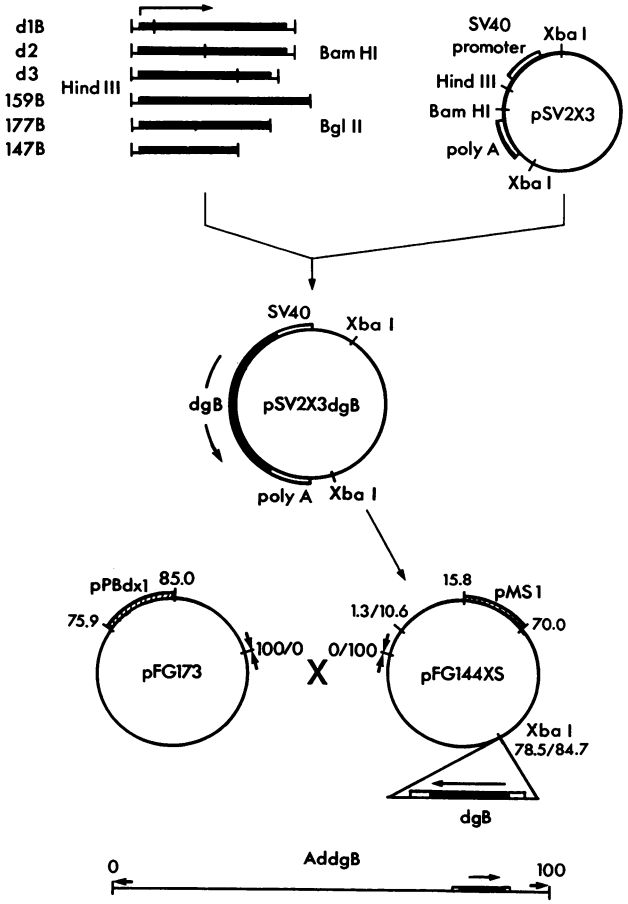


FIG. 2. Strategy for rescue of gB sequences into Ad5. *Hin*dIII-*Bam*HI or *Hin*dIII-*Bgl*II fragments containing mutant forms of the gB gene were inserted into pSV2X3 (46), which contains the SV40 early promoter and polyadenylation site flanked by *Xba*I sites. *Xba*I fragments containing the hybrid SV40-HSV gene were transferred into pFG144XS, a plasmid which contains the rightward sequences of Ad5 (map units 70 to 100) with E3 sequences (map units 78.5 to 84.7) deleted. pFG144XS constructs containing SV40-HSV genes in the left-to-right orientation were selected for rescue into infectious virus. Cotransfection of 293 cells with pFG144XS constructs carrying the chimeric genes and pFG173, which contains the entire Ad5 genome but has a lethal deletion extending into the L4 region of the Ad5 genome, generated the desired recombinant viruses.

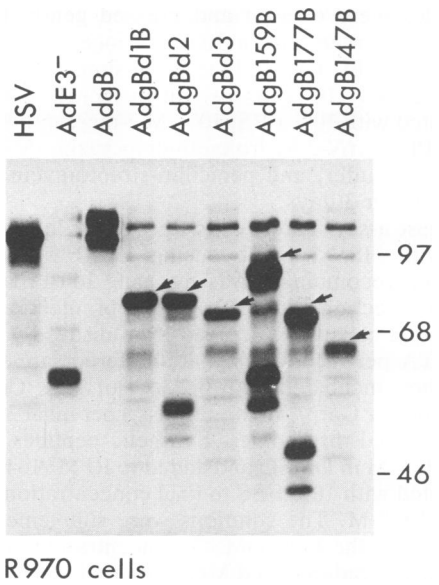


FIG. 3. Expression of mutant forms of gB in cells infected with recombinant Ad vectors. Human R970-5 cells were infected with HSV-1, AdE3⁻, AdgB2 (AdgB), or the following Ad vectors expressing mutant forms of gB: AdgBd1B, AdgBd2, AdgBd3, AdgB159B, AdgB177B, or AdgB147B. Six hours after infection with HSV or 30 to 40 h after infection with Ad vectors, the cells were labeled with [^{35}S]methionine for 2 h. Extracts of the cells were immunoprecipitated by using a pool of gB-specific monoclonal antibodies, and the precipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis. Approximately 10 times the amount of material from cells expressing mutant forms of gB was loaded onto the gels relative to the amount of material from HSV-1 and wild-type AdgB-infected cells. The predicted mobilities of the mutant forms of gB are indicated by arrows, and the positions of molecular mass markers of 97, 68, and 46 kDa are indicated.

map. The resulting pFG144XS constructs containing HSV gB genes were cotransfected with pFG173, which supplies the leftward region of Ad5 DNA (20a), onto monolayers of 293 cells by using the calcium phosphate precipitation technique (24). After 8 to 10 days, individual plaques were picked, virus stocks were produced on 293 cell monolayers, and viral DNA was analyzed by restriction analysis. Recombinant viruses containing intact gB genes were plaque purified twice, and viral DNA was reanalyzed prior to large-scale preparation of virus stocks by using KB cell Spinner cultures.

Radiolabeling, immunoprecipitation, and electrophoresis of mutant glycoproteins. Subconfluent monolayers of R970 cells growing in 35-mm (diameter) dishes (approximately 10^6 cells per dish) were infected with Ad vectors by using 100 PFU per cell or HSV-1 by using 10 PFU per cell. One hour after adsorption at 37°C, the virus inocula were removed and α-MEM supplemented with 2% FBS was added. Thirty to 40 h after infection with Ad vectors (when pronounced CPE was observed) or 5 h after infection with HSV-1, monolayers were washed twice with medium 199 lacking methionine and supplemented with 2% FBS and then incubated with 100 μCi of [^{35}S]methionine per ml. Two hours later, the radioactive medium was discarded and cell extracts were prepared with Nonidet P-40–DOC buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 0.5% sodium deoxycholate [DOC], 1% Nonidet P-40) supplemented with 2 mg of bovine serum albumin and 0.5 mM phenylmethylsulfonyl fluoride and stored overnight at −70°C. Cell lysates were immunoprecipitated by using a pool of anti-gB monoclonal antibodies (15βB2 [29], I-144 [43], I-59 [43], I-252 [43], II-137 [43], and II-387 [43]) followed by incubation with protein A-Sepharose. Immune complexes were washed three times with Nonidet P-40–DOC buffer, eluted with 2× concentrated Laemmli loading buffer (4% sodium dodecyl sulfate [SDS], 20% glycerol, 100 mM Tris [pH 6.8], 0.25% bromophenol blue, 4% 2-mercaptoethanol), and electrophoresed on SDS-polyacrylamide gels cross-linked with 8.5% *N,N*-diallyltartardiamide (27). Gels were infused with 2,5-diphenyloxazole (7), dried, and exposed to a Kodak XAR film.

Generation of anti-HSV CTL. CTL were generated by the protocol of Pfizenmaier et al. (45) with modifications (49). Six- to nine-week-old C57BL/6 (*H-2^b*) or BALB/c (*H-2^d*) mice (Jackson Laboratories, Bar Harbor, Maine) were injected in the hind footpads with 10^6 PFU of HSV-1 F or 5×10^7 PFU of Ad vectors. After 5 days, draining popliteal

lymph nodes were excised and pressed gently through a stainless wire screen with a 10-ml syringe plunger. Viable cells, identified by trypan blue exclusion, were counted; suspended at 4×10^6 cells per ml in RPMI 1640 medium supplemented with 10% FBS, 10^{-5} M 2-mercaptoethanol, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, and penicillin-streptomycin; and cultured at 37°C for 3 days.

^{51}Cr release assay. Target cells were either left uninfected, infected with HSV-1 (F) by using 10 PFU per cell, or infected with recombinant Ads by using 100 PFU per cell. After 6 h of infection with HSV or 24 h of infection with Ad vectors, target cells were scraped from dishes by using 200 mg of EDTA per ml in phosphate-buffered saline, washed with medium, incubated with 0.2 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.) per ml at 37°C for 90 min, and washed three times. Synthetic peptides were dissolved at 10^{-2} M in DMSO and added to RPMI 1640 medium supplemented with 10% FBS to final concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M. The solutions were supplemented with DMSO to keep the final DMSO concentration constant at 1%. Uninfected radiolabeled MC57G target cells were incubated with peptides for 30 min at 37°C. A total of 5×10^4 target cells were mixed with various effector/target ratios in triplicate by using 96-well flat-bottom microtiter plates (Nunclon, Roskilde, Denmark) for 6 h. Percent specific lysis was calculated as described previously (58). In every case, the spontaneous release of ^{51}Cr was less than 30%.

RESULTS

CTL responses to HSV-1 gB are restricted to the K^b MHC gene product and not D^b. In previous experiments, we observed that AdgB-infected target cells were efficiently lysed by CTL from HSV-infected animals in both the *H-2*^{b} and *H-2*^{d} haplotypes but not in the *H-2*^{k} haplotype (61). Lysis of AdgB2-infected cells was often as efficient as or more efficient than lysis of HSV-infected targets in the *H-2*^{b} haplotype, suggesting that gB is a major target for *H-2*^{b}-restricted CTL. Therefore, our initial attempts to map CTL recognition sites in gB involved C57BL/6 mice (*H-2*^{b}). We investigated the restriction of gB-specific CTL with cell lines which express *H-2K*^{b}*D*^{b} (MC57G), *H-2K*^{d}*D*^{b} (HTG), or *H-2K*^{b}*D*^{d} (5R). HSV- or AdgB2-infected MC57G cells, which express both K^b and D^b, were lysed by CTL from C57BL/6 mice, although in this particular experiment lysis of AdgB2-infected cells was lower than with HSV-infected cells (Table 1). Similarly, HSV- and AdgB2-infected 5R cells, which express K^b and D^d, were lysed by C57BL/6 CTL whereas HSV- and AdgB2-infected HTG cells, which express K^d and D^b, were not lysed. These results suggested that recognition of gB by HSV-specific CTL from C57BL/6 mice was restricted by the K^b MHC gene product and not the D^b MHC polypeptide.

Construction of recombinant Ad vectors expressing mutant forms of HSV-1 gB. In order to localize regions of the HSV-1 gB polypeptide which were recognized by HSV-specific CTL, we constructed Ad vectors which expressed a series of mutant gB molecules with internal deletions or C-terminal truncations. Plasmid copies of the HSV-1 gB gene, in which the gene was mutated by insertion of oligonucleotide linkers, were previously described (12). Three plasmids, pKd1B, pKd2, and pKd3, containing internal deletions were constructed by removing sequences between inserted oligonucleotide linkers (Fig. 1). Three additional plasmids, pK159B, pK177B, and pK147B, containing *Bgl*II oligonucleotide linkers inserted internally in the gB gene were used to delete 3′ sequences prior to insertion into Ad vectors. These six mutations collectively remove all of the coding sequences of HSV-1 gB except residues 1 to 43, which primarily encode the 30-residue signal sequence which is removed from the mature protein (13). *Hin*dIII-*Bam*HI or *Hin*dIII-*Bgl*II fragments containing the mutant gB genes were inserted between the SV40 early promoter and polyadenylation signal in pSV2X3 (Fig. 2). The hybrid SV40-HSV gene was inserted into the nonessential E3 region of Ad5 by using pFG144XS. Ad vectors carrying the SV40-HSV sequences were then rescued into infectious Ad5 by cotransfecting 293 cells with pFG144XS plasmids carrying the mutant gB genes and pFG173, which supplies essential Ad genes. Recombinant viruses derived in this manner are replication competent in human cells and routinely express the inserted gene in both human and murine cells (22).

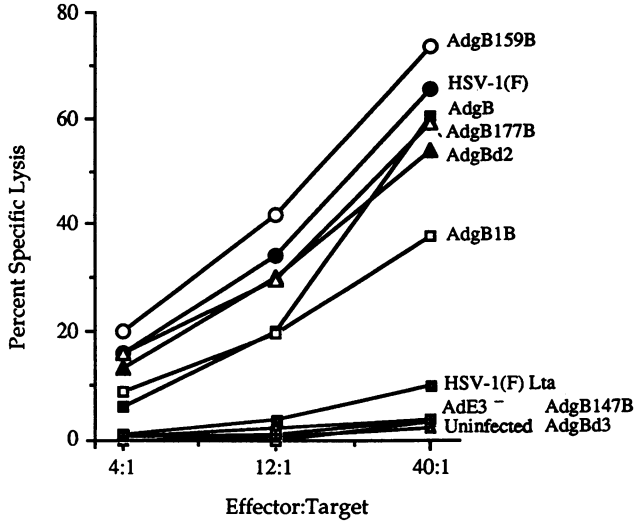


FIG. 4. HSV-specific CTL lysis of target cells infected with Ad vectors expressing mutant forms of gB. MC57G cells were left uninfected (▲) or were infected with HSV-1 (●), AdgB (AdgB2) (■), AdE3$^-$ (⊞), or Ad vectors expressing the following mutant forms of gB: AdgBd1B (□), AdgBd2 (▲), AdgBd3 (◇), AdgB159B (○), AdgB177B (△), or AdgB147B (⦿). Lta (*H-2*^{k}) cells infected with HSV-1 (■) served as an *H-2* mismatched control. (Lysis of cells infected with AdgB147B and AdgBd3 was very similar to that observed with AdE3$^-$-infected and uninfected cells. Thus, symbols indicating the AdgBd3 and AdgB147B values are obscured by those for uninfected and AdE3$^-$ controls, respectively.) After 6 h in the case of cells infected with HSV-1 or after 24 h when cells were infected with Ad vectors, the target cells were labeled with ^{51}Cr for 90 min and washed three times. Bulk, primary CTL from C57BL/6 (*H-2*^{b}) mice infected with HSV-1 were incubated for 6 h with target cells at various effector/target cell ratios in triplicate by using flat-bottom microtiter plates. Percent specific lysis was calculated as described in Materials and Methods.

Expression of gB by recombinant Ad vectors. To confirm that the recombinant Ad vectors express the mutant gB polypeptides, human R970-5 cells were infected with AdgB2, which expresses wild-type gB (29), or the Ad vectors expressing mutant gB proteins. Then infected cells were labeled with [^{35}S]methionine and radiolabeled gB was immunoprecipitated by using a pool of gB-specific monoclonal antibodies. The immunoprecipitated proteins were electrophoresed in SDS-polyacrylamide gels, and in every case a polypeptide of the expected molecular weight was detected

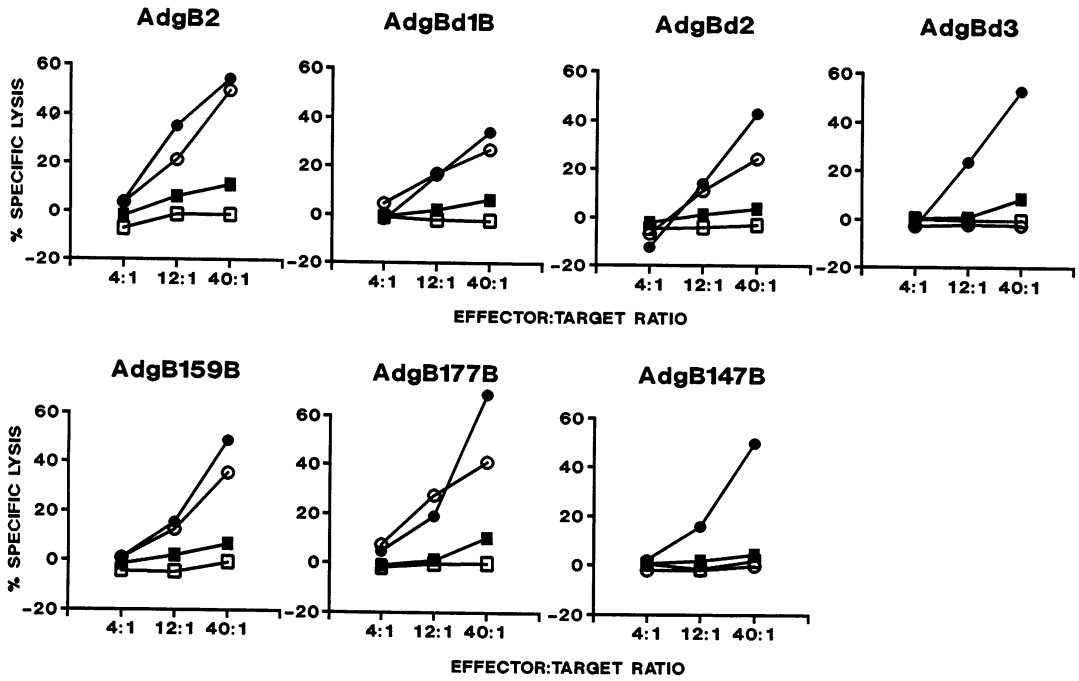


FIG. 5. Lysis of HSV-infected target cells by CTL from mice infected with Ad vectors expressing mutant forms of gB. C57BL/6 mice were infected in the hind footpads with 5×10^7 PFU of AdgB2, which expresses wild-type gB, or the following Ad vectors expressing mutant forms of gB: AdgBd1B, AdgBd2, AdgBd3, AdgB159B, AdgB177B, or AdgB147B. After 5 days, draining lymph nodes were removed and lymphocyte cultures were prepared. The lymphocytes were suspended at 4×10^6 cells per ml and incubated for 3 days. Target MC57G cells left uninfected (□) or infected with HSV-1 (○) or with AdgB2 (●) or Lta cells infected with HSV-1 (■) were labeled with ^{51}Cr and mixed with CTL at various effector/target cell ratios in triplicate. The assay was incubated for 6 h, and the percent specific ^{51}Cr release was determined.

in lysates of cells infected with the Ad vectors (Fig. 3). Faster-migrating protein species were also detected with some of the mutant gB molecules. One of these faster-migrating species in AdgB159B-infected cells may be related to an Ad protein of approximately 58 kDa which was detected in a variable fashion in previous experiments (29) and observed in $AdE3^-$-infected cells in this experiment. Expression of gB in AdgB2-infected cells was relatively high compared with that observed in HSV-infected cells (29). However, cells infected with Ad vectors expressing mutant gB polypeptides expressed 10-fold-lower levels of gB, and the proteins appeared much less stable. Instability of mutant gB proteins may account for reduced levels of protein and the faster-migrating species observed in Fig. 3, as these probably include proteolytic fragments.

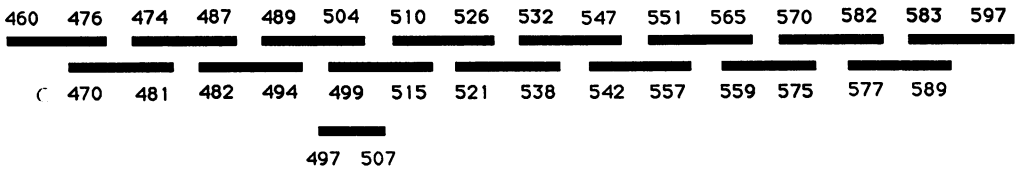
The single band observed in the HSV-infected cells (Fig. 3) appears to indicate that the immature form of gB, pgB, was not converted to mature gB during the relatively short labeling period used in this experiment. Immature forms of gB are normally processed slowly to mature forms of gB in HSV-infected cells, i.e., ≥3 h. We have observed that the maturation of gB in AdgB-infected cells is often more rapid than in HSV-infected cells (25a), consistent with observations made with other HSV glycoproteins in transfected cells (30). It also appears that mutant forms of gB did not undergo significant levels of posttranslational maturation, because the doublet of two closely migrating species observed in AdgB2-infected cells was not observed with mutant gB molecules. This is not unexpected, because mutant forms of HSV membrane glycoproteins, where significant deletions have been made, are often not transported to the cell surface and are not fully processed (17).

CTL lysis of cells infected with Ad vectors expressing mutant gB molecules. To determine whether mutant forms of gB were recognized by HSV-specific, *H-2*^{b}-restricted CTL, ^{51}Cr release assays were performed with lymphocytes from HSV-infected C57BL/6 mice and MC57G target cells infected with the various Ad vectors. As observed previously (61), target cells infected with AdgB2 were lysed with an efficiency similar to that observed for HSV-infected cells (Fig. 4). Cells infected with four of the Ad vectors, AdgB159B, AdgB177B, AdgBd2, and AdgBd1B, were also efficiently lysed by the HSV-specific CTL, whereas cells infected with AdgB147B and AdgBd3 were not lysed. Furthermore, *H-2* mismatched controls, Lta cells (*H-2*^{k}) infected with HSV, were not lysed. The observation that

TABLE 2. Lysis of target cells incubated with synthetic peptides[a]

Peptide	Residues	% Specific lysis		
		10^{-4} M	10^{-5} M	10^{-6} M
None		0	0	0
1	460–476	3	0	2
5	489–504	42	17	9
6	499–515	46	40	39
5/6	497–507	47	42	37
7	510–526	4	1	2

[a] ^{51}Cr-labeled MC57G target cells were incubated with the indicated peptide at 10^{-4}, 10^{-5}, or 10^{-6} M for 30 min at 37°C and incubated for 6 h with lymphocytes from HSV-infected C57BL/6 mice by using an effector/target cell ratio of 40:1. Specific release of ^{51}Cr was calculated as described in Materials and Methods.



HSV-1 61

peptide		10^{-4} M		10^{-5} M		10^{-6} M	
CTL		-	+	-	+	-	+
1	460-476	0	4	2	0	4	0
2	470-481	1	**19**	4	6	3	3
3	474-487	3	4	1	4	0	2
4	482-494	3	7	0	5	3	2
5	489-504	3	**49**	2	**21**	3	6
6	499-515	4	**43**	3	**46**	3	**47**
7	510-526	0	3	1	3	1	2
8	521-538	5	7	0	2	0	2
9	532-547	0	1	1	4	2	3
10	542-557	1	2	2	2	1	3
11	551-565	2	5	2	6	1	4
12	559-575	16	18	2	4	0	2
13	570-582	1	2	4	4	3	4
14	577-589	0	0	3	0	0	2
15	583-597	7	9	1	2	1	4

FIG. 6. Synthetic peptides recognized by HSV-specific CTL. Fifteen synthetic peptides 12 to 19 residues in length, overlapping by 6 to 8 residues, and spanning residues 460 to 597 were synthesized. MC57G cells were labeled with ^{51}Cr and treated with 10^{-4}, 10^{-5}, or 10^{-6} M peptide for 30 min at 37°C. The cells were then incubated for 6 h with lymphocytes from HSV-infected C57BL/6 mice by using an effector/target cell ratio of 40:1. Under these conditions, the HSV-specific CTL released 61% of the ^{51}Cr from HSV-infected cells. In each case, the ^{51}Cr release assays were performed in the absence (−) or presence (+) of effector cells to control for cytotoxic effects of the peptides. An 11-residue peptide denoted 5/6 (497 to 507) which contains the region shared by peptides 5 (489 to 504) and 6 (499 to 515) was subsequently synthesized to determine whether the overlapping region was recognized by anti-HSV CTL (Table 2).

HSV-specific CTL failed to recognize mutant d3, which lacks residues 463 to 710, yet recognizes 177B, which is truncated at residue 594, suggested that the major CTL site(s) in gB lie between residues 462 and 594 (Fig. 1). This conclusion was further supported by observations that (i) mutant 147B, which lacks residues downstream of residue 379, was not recognized by CTL and (ii) mutants 159B, d1B, and d2, which all include residues 462 to 594, were recognized.

Lysis of HSV-infected target cells by CTL from mice infected with Ad vectors expressing mutant forms of gB. Previously, we observed that lymphocytes removed from *H-2*^{b} or *H-2*^{d} mice inoculated with AdgB2 were able to lyse HSV-infected target cells (25b). In order to confirm and extend our observations on CTL sites in gB, we injected C57BL/6 (*H-2*^{b}) mice with Ad vectors expressing mutant gB molecules and tested CTL from the animals in assays involving HSV-infected or AdgB2-infected target cells (Fig. 5). The CTL preparations from animals infected with various Ad vectors differed somewhat in their ability to lyse AdgB2-infected target cells (Fig. 5), suggesting varying quantities of CTL in the lymphocyte cultures. However, it was clear that AdgBd1B-, AdgBd2-, AdgB177B-, and AdgB159B-infected mice lysed HSV-infected targets (Fig. 5), whereas CTL from AdgB147B- and AdgBd3-infected mice did not. Therefore, mutant forms of gB with the region between residues 462 and 594 deleted (Fig. 1) were unable to stimulate anti-HSV CTL. These observations further support the hypothesis that the major CTL site(s) in gB lie in this region.

Synthetic peptides localize the major CTL site in gB to residues 497 to 507. It is well known that MHC class I polypeptides can present exogenously added peptides to CTL, which can then lyse the target cells. To further localize the T-cell site(s) within the 131-amino-acid region defined by using gB mutants, overlapping peptides were synthesized spanning the entire region (Fig. 6). The peptides were 12 to 19 residues in length and overlapped by 6 to 8 residues. MC57G (*H-2*^{b}) cells were treated with 10^{-4} M, 10^{-5} M, or 10^{-6} M peptide at 37°C for 30 min and incubated with lymphocytes from HSV-infected C57BL/6 (*H-2*^{b}) mice, which in this assay were able to release 61% of the ^{51}Cr from HSV-infected target cells. To control for cytotoxic effects of the peptides, target cells pretreated with peptides were also incubated in the absence of effector cells. Two peptides, 8 and 12, showed cytotoxic effects at a concentration of 10^{-4} M in some, but not all, assays and were not completely soluble in aqueous medium. For example, peptide 12 caused release of 15% of the ^{51}Cr in the absence of CTL (Fig. 6). Cells treated with peptide 5 (residues 489 to 504) or peptide 6 (499 to 515) were consistently lysed by primary HSV-specific CTL (Fig. 6). With peptide 6, CTL lysis was observed at concentrations down to 10^{-6} M in this assay and as low as 10^{-7} M in another assay (results not shown). In contrast, peptide 5 was less effective at lower concentrations. In some assays, peptide 2 or peptide 9 sensitized cells to CTL-induced lysis. However, these peptides did not produce consistent results and the levels of cell lysis were not substantial, especially at lower peptide concentrations.

Peptides 5 (residues 489 to 504) and 6 (499 to 515) overlap by six amino acids. Since it is known that peptides as short as five or six residues can be recognized by T cells in conjunction with MHC class I polypeptides (60), we inves-

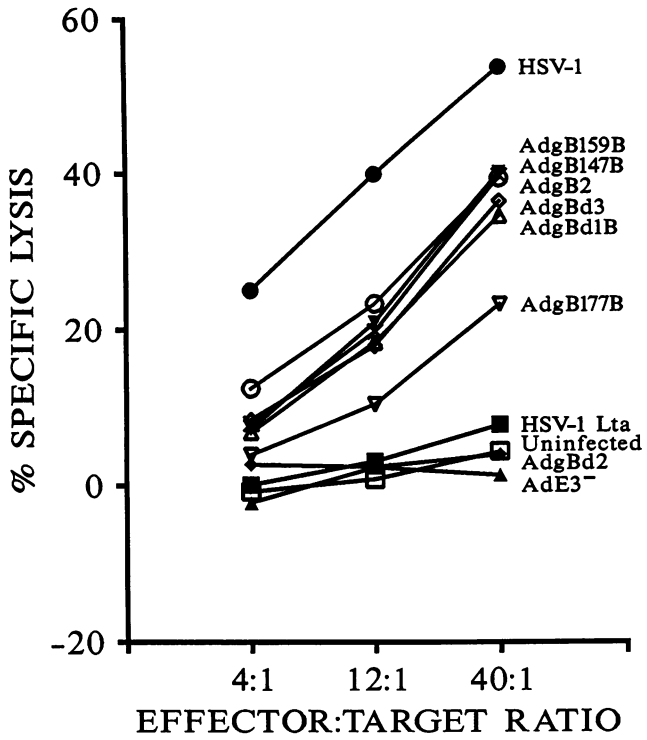


FIG. 7. Lysis of cells expressing mutant forms of gB by *H-2*^{d}-restricted anti-HSV CTL. BALB/c (*H-2*^{d}) mice were infected with HSV-1, and 5 days later, draining lymph nodes were removed and cultured for 3 days. SVBALB (*H-2*^{d}) target cells were left uninfected (□) or were infected with HSV-1 (●), AdE3⁻ (▲), AdgB2 (○), AdgBd1B (△), AdgBd2 (◆), AdgBd3 (◇), AdgB159B (▼), AdgB177B (▽), or AdgB147B (X). Lta cells infected with HSV-1 (■) served as an *H-2* mismatched control. Target cells were labeled with ^{51}Cr for 90 min and mixed with effector cells at various effector/target cell ratios for 6 h. Percent specific lysis was determined as described in Materials and Methods.

tigated the possibility that the sequences shared by these two peptides formed part of the recognition site for the HSV-specific CTL. An 11-residue peptide designated 5/6 (residues 497 to 507; TSSIEFARLQF) spanning the overlap region was synthesized and was efficiently recognized by HSV-specific CTL (Table 2). Although the minimum sequences required for recognition have not been established, it appears that the 11-residue peptide consisting of residues 497 to 507 contains all of the essential amino acids required for recognition of this CTL site. Together, the experiments involving mutant forms of gB and those involving synthetic peptides have allowed us to localize an immunodominant CTL site recognized by *H-2*^{b}-restricted CTL to a region in gB within residues 497 to 507.

Recognition of mutant gB molecules by *H-2*^{d}-restricted CTL. In order to begin to identify CTL sites in gB recognized by *H-2*^{d}-restricted CTL, we also tested *H-2*^{d} target cells infected with Ad vectors expressing mutant gB molecules in ^{51}Cr release assays. SVBALB (*H-2*^{d}) cells infected with AdgB2 were lysed by primary HSV-specific CTL from BALB/c (*H-2*^{d}) mice, although lysis was less complete than with HSV-infected targets (Fig. 7), as we found previously (61). Similarly, targets infected with AdgBd1B, AdgBd3, AdgB159B, AdgB177B, and AdgB147B were all lysed by anti-HSV CTL. In contrast, AdgBd2-infected cells were lysed only at levels comparable to uninfected cells. This observation, coupled with the finding that AdgB147B-infected cells were efficiently lysed, suggested that *H-2*^{d}-restricted CTL recognize an immunodominant site in gB between residues 233 and 379 (Fig. 1). In the experiment shown in Fig. 7, lysis of AdgB177B-infected targets was fractionally lower than lysis observed with AdgB2- or AdgB147B-infected cells. However, since 147B (which was truncated so that all of the sequences deleted in 177B are also deleted) is efficiently lysed by anti-HSV CTL, the lower level of lysis of 177B-infected cells was most probably not due to deletion of a CTL site and may represent reduced levels of expression of the 177B mutant protein.

DISCUSSION

An immunodominant site in HSV-1 gB recognized by *H-2*^{b}-restricted anti-HSV CTL was identified initially by using recombinant Ad vectors to express deletion mutants of the protein. Analysis of CTL responses to the mutant proteins suggested that the CTL site(s) was contained in a 131-amino-acid region of gB between residues 462 and 594. Subsequent experiments utilizing overlapping synthetic peptides identified an 11-amino-acid region including residues 497 to 507 which appears to contain a single T-cell recognition site. The 11-residue peptide is apparently presented exclusively by the K^b MHC gene product and not the D^b polypeptide, suggesting that this peptide may bind to K^b and not D^b. Our results are consistent with the observation that primary HSV-specific CTL derived from C57BL/6 mice were restricted primarily to the K^b class I MHC gene product with little or no response restricted by the D^b gene product (28).

A large fraction of gB-specific CTL apparently recognize the site identified in these studies, because deletion mutations affecting the site abolished recognition by anti-HSV CTL and only peptides including residues 499 to 507 were able to sensitize cells to CTL lysis when the peptides were used at micromolar concentrations. Furthermore, CTL from animals immunized with gB mutants lacking the site were unable to recognize HSV-infected cells. The observation that a single site in gB was recognized by HSV-specific CTL is particularly noteworthy, because the CTL in these experiments were bulk, primary-induced CTL which were not restimulated in vitro. Many other examples of CTL sites identified in viral polypeptides have involved restimulated lymphocyte populations or CTL clones. In these cases, it is more difficult to discern the relative importance of CTL responses to the defined site in question, since in vitro restimulation could cause skewing of the relative responses to individual viral proteins or CTL sites.

The CTL site in gB recognized by *H-2*^{b}-restricted CTL is currently defined as an 11-residue peptide and may not be the minimal or optimum peptide which can be recognized. Studies are under way to establish the minimum sequences which are required for optimal recognition. During our attempts to define CTL sites in gB, we did not make use of any of the algorithms which have been used to predict CTL recognition domains (14, 51, 53). These algorithms predicted a considerable number of peptides throughout this large protein and, in addition, the algorithms failed to predict several recently defined CTL sites (42, 60). After identifying the CTL site, it was found that the central region of the 11-mer contains a sequence, Glu–Phe–Ala–Arg-504, which fits the prediction of two charged or polar residues separated by two or three hydrophobic residues (51). However, the

11-residue peptide did not contain an amphipathic α helix, which would have been predicted by the other principal algorithm (14, 53).

The amino acid sequences in gB surrounding and including the CTL recognition site are highly conserved in three different strains of HSV-1 and HSV-2. Residues 489 to 515, which include sequences upstream and downstream of the 11-residue CTL recognition site, were 100% homologous in three different HSV-1 strains and one HSV-2 strain (10, 11, 37, 44). Therefore, we would expect that HSV-specific, $H\text{-}2^b$-restricted CTL would recognize many HSV-1 and HSV-2 strains by means of this immunodominant site, and our unpublished results support this view.

Preliminary efforts to localize sites in gB recognized by $H\text{-}2^d$-restricted CTL suggest that a different region of the molecule is recognized. Mutant d2, from which residues 233 to 463 were deleted, was inefficiently recognized by $H\text{-}2^d$-restricted anti-HSV CTL, whereas mutant 147B, which is truncated at residue 379, was recognized. Therefore, an immunodominant site (or sites) in gB for $H\text{-}2^d$ CTL is closer to the N terminus of the protein, between residues 233 and 379. Furthermore, gB was not recognized by $H\text{-}2^k$-restricted CTL (61). Thus, as has been found in other systems, the CTL site defined for $H\text{-}2^b$ restriction is not recognized by CTL of at least two other haplotypes.

The CTL recognition site in gB defined in these studies is the only CTL recognition site described to date for any of the members of the alphaherpesvirus family. CTL sites have been identified for members of the betaherpesvirus (murine cytomegalovirus; 16) and gammaherpesvirus (EBV; 8, 39) families. However, as was mentioned in the Introduction, these viruses establish infections in which a small subset of viral gene products is expressed for extended periods of time, thereby greatly limiting the number of potential target antigens. In contrast, the infectious cycles of alphaherpesviruses are relatively short and many viral gene products can be detected within a few hours of infection and throughout most of the infection. Thus, efforts to identify targets for HSV-specific CTL have been complicated by the large numbers of viral proteins and potential sites within these proteins. The situation is further complicated by difficulties in studying HSV CTL responses in the natural host, where haplotypes are numerous and where cytotoxic responses involving peripheral blood lymphocytes are relatively weak and masked by natural killer activities. Nevertheless, at least in this murine system, gB is recognized by a surprisingly large fraction of HSV-specific CTL and acts as an excellent target for anti-HSV CTL. The identification of this CTL site in gB should facilitate studies on the role of CTL responses to defined antigens in murine models of HSV infection. In addition, it is hoped that synthetic peptides should be useful in establishing and characterizing stable anti-HSV CTL clones, which have been difficult to establish to date.

Animals inoculated with the Ad vector, AdgB2, were protected from a subsequent challenge with a lethal dose of HSV-2 (36). In these studies, gB-specific antibodies were detected and these antibodies were complement dependent and not present at high levels. Here, we have shown that lymphocytes from animals inoculated with AdgB2 could efficiently lyse HSV-infected target cells and that it was necessary to include the CTL recognition site in order to affect lysis of HSV-infected targets. The Ad vectors described in this study should be useful in defining the relative contribution of CTL to protective responses to HSV, and, in addition, it may be possible to protect animals with an Ad vector which expresses the isolated CTL recognition site. Furthermore, the role of CTL in the establishment of latency may also be analyzed with these Ad vectors. The studies described here further demonstrate the utility of Ad vectors in the analysis of immune responses to individual viral polypeptides. Most other studies of this type have relied on the use of vaccinia virus vectors, which hold great potential as viral vaccines. Ad vectors also have many advantages as potential vaccine vehicles (for a review, see reference 22) and should be strongly considered as alternatives to vaccinia virus.

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