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I. Objectives of This Review

Cytotoxic CD8 T lymphocytes (CTLs) are critical for the clearance of noncytopathic viruses from infected cells (Zinkernagel, 1996). Once it became evident that CTLs were indeed important for virus clearance, it was recognized that virus persistence must include the ability to evade this arm of the immune response. Several mechanisms by which viruses are able to diminish, delay, or prevent recognition by CTLs have been described (Oldstone, 1991; Ploegh, 1998). This review will concentrate on one mechanism used by viruses to persist—namely, the selection of a variant virus in which changes in the sequence of a CTL epitope abrogate recognition. In the first part of this review, the unique features of cytotoxic CD8 T cell function in the central nervous system (CNS) will be discussed. In the second part, the role of CTL escape mutants in viral evasion of the immune system and subsequent disease progression in non-CNS infections will be summarized. Finally, evidence showing that CTL escape mutants play a key role in virus amplification and the development of clinical disease in at least one model of virus-induced demyelination will be described in detail.
II. INTRODUCTION

A. Aspects of Immunological Response in the CNS

The immune response in the CNS is similar to the response in extraneural tissue, but several aspects of activation of the immune response, cellular trafficking, and antigen presentation are unique to the CNS (reviewed in Lassmann, 1997; Lassmann et al., 1991; Matyszak, 1998; Williams and Hickey, 1995). The function of cytotoxic CD8 T cells in the inflamed CNS and the major histocompatibility complex (MHC) class I antigen expression in this tissue are most pertinent for the topic of this review and will be preferentially discussed in this section.

Although the CNS has classically been considered a site of immune privilege, surveillance of the normal CNS by circulating, activated lymphocytes occurs, with a limited number of lymphocytes being present in the normal CNS at any given time (Hickey, Hsu, and Kimura, 1991). However, considerable data suggest that the immune response in the CNS is initiated not in the parenchyma but, rather, in draining lymph nodes located in the deep cervical tissue (Cserr and Knopf, 1992). Dendritic cells, critical for antigen presentation, are virtually absent from the normal CNS, suggesting that virus antigen must spread to the draining lymph node tissue before activation can occur (Hart and Fabre, 1981; Matyszak, 1998; Matyszak and Perry, 1996). In support of this contention, influenza virus inoculated directly into the CNS parenchyma does not induce an immune response until virus spreads to the cerebrospinal fluid (CSF) and, soon thereafter, to the draining lymph nodes (Stevenson et al., 1997a; Stevenson et al., 1997b). Once the immune response has been initiated, breakdown of the blood–brain barrier, presence of plasma proteins, and recruitment of leukocytes characterize the CNS inflammatory response. Notably, cells with the phenotype of dendritic cells are detected as part of the cellular infiltrate in inflammatory processes and are believed to contribute to sustaining the immune response during chronic inflammation (Matyszak, 1998; Matyszak and Perry, 1996).

Within the CNS, CD8 and CD4 T cell effector functions are virtually the same as in peripheral sites of inflammation. CD8 T cells are responsible for the majority of cytotoxic activity and exhibit three effector mechanisms: perforin/granzyme-mediated killing; Fas/Fas ligand-mediated killing; and cytokine secretion (Liu, Young, and Young, 1996; Smyth and Trapani, 1998). In one well-documented example, perforin is critical for the CD8 T cell response to LCMV, both peripherally and in the CNS (Kagi et al., 1994). Fas/FasL interactions have not been documented to be critical for CD8 T cell effector function in the CNS, but FasL expression by infiltrating CD4 T cells has been impli-
cated in oligodendrocyte death in inflammatory settings such as experimental allergic encephalomyelitis (EAE) and multiple sclerosis (MS) (D'Souza et al., 1996; Sabelko et al., 1997; Waldner et al., 1997). Secretion of interferon-γ (IFN-γ) and of other cytokines are of critical importance in orchestrating multiple components of the cellular and humoral immune responses. Furthermore, in some cases, cytokines are capable of directly inhibiting viral replication. For example, IFN-γ appears to be critical for virus clearance from infected oligodendrocytes in mice infected with mouse hepatitis virus (Parra et al., 1999). In another example, genetic disruption of IFN-γ or its receptor inhibits virus clearance in strains of mice normally resistant to infection with Theiler's murine encephalomyelitis virus (TMEV) (Fiette et al., 1995).

CTLs also release chemokines through granule exocytosis, thereby sustaining the immune response by contributing to the influx of other inflammatory cells (reviewed in Baggiolini, Dewald, and Moser, 1997; Luster, 1998). The influx of both antigen-specific and nonspecific cells may contribute to virus clearance but also has the potential for increasing damage to nearby uninfected cells (bystander damage). This mechanism has been postulated to be significant in the pathogenesis of several CNS diseases with inflammatory components, including demyelination in animals with virus-induced or autoimmune demyelination (Houtman and Fleming, 1996b; Lassmann, 1997).

One major difference between the CNS and extraneural tissue is found in MHC class I antigen expression. In extraneural tissue, MHC class I antigen is expressed constitutively by most cells. However, in the uninflamed CNS, constitutive expression of MHC class I and II antigen is infrequent and is confined to a subset of endothelial cells (MHC class I) and macrophages/microglia (MHC class II) (reviewed in Lampson, 1995; Shrikant and Benveniste, 1996). Expression of these molecules by astrocytes, oligodendrocytes, or neurons is not believed to occur in the normal CNS. However, this conclusion may need to be modified since, in a recent study, the coordinated expression of MHC class I antigen (heavy chain and β2-microglobulin) and of a T cell receptor component (CD3ζ) by neurons during development was postulated to be important for synapse formation (Corriveau, Huh, and Shatz, 1998).

MHC class I and class II expression are upregulated in the CNS in multiple pathological settings with infectious or noninfectious etiologies (also reviewed in Lampson, 1995; Shrikant and Benveniste, 1996). As discussed above, MHC class I expression by antigen-presenting cells resident in the CNS is not involved in initiation of the CTL response but is likely to be critical for its perpetuation. Expression of MHC class I molecules by infected cells is also required for recognition and lysis by activated antigen-specific CD8 T cells. The most convincing data show
that MHC class I and II antigens are upregulated on microglia/macrophages in inflammatory diseases of the CNS, including MS and TMEV- and MHV-induced demyelinating encephalomyelitis. T cell costimulatory molecules such as B7-1 and B7-2 are also upregulated on these cells and, in some cases, activated microglia/macrophages isolated from the CNS can directly stimulate antigen-specific T cells proliferation (Bo et al., 1994; Matyszak, 1998; Pope et al., 1998; Xue et al., 1999). MHC class I and class II expression by astrocytes and oligodendrocytes has been demonstrated in vitro, although, in some cases, only after exposure to proinflammatory cytokines, such as interferon-γ (Lampson, 1995; Shrikant and Benveniste, 1996). Whether astrocytes and oligodendrocytes upregulate MHC class I and class II expression in the inflamed CNS remains quite controversial, with expression of these molecules being reported in some studies (Lampson, 1995; Shrikant and Benveniste, 1996). In most recent studies, MHC antigen expression by astrocytes and oligodendrocytes in inflamed tissue was not detected (e.g., Pope et al., 1998; Xue et al., 1999), but these negative conclusions must be tempered because a biologically significant level of expression might not be detectable with presently available assays. MHC class I antigen upregulation on neurons has not been reported in pathological conditions, although a recent set of elegant studies demonstrated the expression of these molecules following inhibition of electrical activity (Neumann et al., 1995; Neumann et al., 1997). If verified in the infected CNS, these results would suggest that only neurons sufficiently damaged by infection with a virus or another infectious agent would be recognized by antigen-specific CTLs. Furthermore, this mechanism would minimize destruction of infected, but still functionally active, neurons.

Although there is no agreement on the specifics, these studies clearly demonstrate that MHC class I and II antigens are expressed on CNS-derived cells in vitro and in vivo during inflammatory processes. As a consequence, they provide a framework for interpreting experiments that describe the selection of CTL escape mutants in the CNS of virus-infected mice.

B. Evidence for Selection of CTL Escape Mutants in Infections Occurring Outside the CNS

The first experimental evidence for virus escape from CTL recognition by mutation of a CD8 T cell epitope was provided by Pircher et al., (1990). In mice that were transgenic for a T cell receptor (TCR) specific for lymphocytic choriomeningitis virus (LCMV), infection with LCMV resulted in the rapid appearance of virus mutated in the target CTL epitope. Infection of TCR transgenic mice, but not of immunocompe-
tent B6 mice, with mutant LCMV resulted in a delay in the kinetics of virus clearance. Similar results were obtained when wild-type mice were infected with LCMV variants in which some or all of the appropriate CTL epitopes were mutated (Lewicki et al., 1995; Moskophidis and Zinkernagel, 1995). Notably, LCMV escape mutants are not generated in immunocompetent mice after infection with wild-type virus. In these mice, CTLs recognizing multiple epitopes are detected, suggesting that an immune response directed against several epitopes precluded the selection of CTL escape mutants, at least in settings in which virus clearance was relatively efficient.

A subsequent study suggested that CTL escape mutants may emerge and become the predominant virus in human populations in which a single HLA allele is overrepresented, and in which an immunodominant epitope is restricted by that allele. The strain of Epstein-Barr virus (EBV) circulating in humans residing in the coastal regions of Papua New Guinea contains a mutation in an immunodominant CD8 T cell epitope that diminished binding to the HLA-All molecule (De Campos-Lima et al., 1994). HLA-A11 is present at a high frequency in this population, suggesting that the selection of virus encoding this mutation was immune-driven. However, this conclusion may not be warranted since the same mutation is found in virus circulating in the highland populations of Papua New Guinea, even though the HLA-A11 allele is not overrepresented in that group (Burrows et al., 1996).

The studies of LCMV-infected mice suggested that efficient virus clearance precluded the selection of CTL escape mutants. Conversely, less efficient virus clearance would be predicted to facilitate the emergence of these mutants. Therefore, the potential contribution of CTL mutants to pathogenesis was examined next in several persistent infections, including human and nonhuman primates chronically infected with the human immunodeficiency virus (HIV-1), hepatitis B virus (HBV), and hepatitis C virus. The emergence of CTL escape mutants during the course of persistence was documented in each of these infections (Franco et al., 1995; McMichael and Phillips, 1997; Weiner et al., 1995). The most convincing data suggesting a role for these variants in disease progression comes from studies in which the emergence of CTL escape mutants are documented early during the infection. Selection at early times postinfection is consistent with a role in delaying the elimination of virus from the infected host. In one study, Borrow et al. (1997) described an HIV-infected patient in which the initial CTL response was directed at a single HLA B44-restricted epitope. Early in the course of the infection, a mutation in a presumptive anchor residue was detected, leading to a loss of recognition by the patient's CTLs. Shortly thereafter, the CTL response spread to addi-
tional, presumably less immunodominant, epitopes. These responses were able to control the virus for a short period, but were, ultimately, inadequate because the patient exhibited a rapid rate of disease progression with a fatal outcome. In another study, mutations resulting in CTL escape were identified in the nef gene in a patient during primary HIV infection. These mutations abrogated recognition by nef-specific CTLs harvested from the patient, suggesting that their selection was also immune-driven and that it contributed to virus persistence (Price et al., 1997).

In still another study, CTL escape mutants were identified in HIV-infected patients several years after the primary infection, coincident with increased virus replication and decreased CD4 T cell counts (Goulder et al., 1997). It is uncertain, in these cases, whether CTL escape caused disease progression or whether it was a consequence of increasingly ineffectual control of virus replication by other components of the immune system, such as antibodies and CD4 T cells. CTL escape mutants that emerge only after immune surveillance has diminished may still contribute to disease progression in HIV-infected patients, even if they are not the primary cause for the loss of control by the immune system. CTL escape mutants were also selected in an HIV-infected individual treated with CTLs directed against a single epitope (Koenig et al., 1995), thereby showing that, as in LCMV-infected TCR transgenic mice, a strong, monospecific CTL response facilitated their appearance.

CTL escape variants are occasionally detected in patients chronically infected with HBV, usually in the context of a narrowly focused, strong immune response (Bertoletti et al., 1994a; Bertoletti et al., 1994b). A more common finding in patients infected chronically with HBV is the lack of a significant CTL response (Rehermann et al., 1995), suggesting that variation in HBV-specific T cell epitopes is important in only a minority of patients. Similarly, CTL escape mutants were detected in a chimpanzee chronically infected with hepatitis C virus (Weiner et al., 1995), but persistence appears to be associated, most commonly, with a weak CTL response (Rehermann et al., 1996).

C. Mechanisms of CTL Escape

Mutations in an epitope or in its flanking sequences can result in escape from surveillance by CD8 T cells, by affecting any one of several steps along the pathway used to generate peptides for presentation by MHC class I molecules. Antigen processing involves proteolytic cleav-
age by proteosomes in the cytoplasm and transport of the resulting peptides to the endoplasmic reticulum for binding to the MHC class I molecule. Mutations in sequences flanking a CTL epitope may affect proteolytic cleavage or transport into the endoplasmic reticulum. Only a few examples of mutations affecting either of these processing steps have been described (Eisenlohr, Yewdell, and Bennink, 1992; Ossendorp et al., 1996). In one example, comparison of the sequence of an immunodominant CTL epitope encoded by two related murine leukemia virus families (AKV/MCF and Friend/Moloney/Rauscher [FMR]) showed that a single amino acid change present in the FMR strains abrogated CD8 T cell recognition (Ossendorp et al., 1996). The mutation did not affect binding of peptide to the MHC class I molecule or immunogenicity of a peptide corresponding to the variant epitope. Rather, the loss of recognition occurred only after endogenous processing of antigen and resulted from alterations in proteosomal processing, which were shown using purified 20S proteosomes in vitro. These conclusions assume that processing in vitro mimics what occurs in the cell and that other differences in the amino acid sequence between the two viruses did not contribute to the observed differences. Mutations in sequences flanking a CTL epitope are difficult to detect, and therefore may be more important in CTL escape than is appreciated at present.

More commonly, mutations in CTL epitopes that affect binding to the MHC molecule, or affect recognition by the TCR, have been identified. Some of the mutations described above, in HIV-1 infected individuals, diminish binding to the MHC class I molecule or result in increased rates of dissociation from the MHC molecule (reviewed in McMichael and Phillips, 1997). Mutations that impair binding to the MHC molecule are the simplest to interpret and, depending on the diminution of binding, will completely abrogate recognition by epitope-specific CD8 T cells. Mutations that affected binding to TCRs were first reported in studies describing selection of LCMV CTL escape mutants in mice transgenic for an LCMV-specific TCR (Pircher et al., 1990). Peptides mutated in residues that directly contact the TCR have also been described in virus isolated from HIV-infected patients (McMichael and Phillips, 1997). These mutations may result in partial or complete loss of recognition by autologous CTLs.

During the course of these studies on viral variation, mutations were identified in viral RNA and DNA isolated from patients persistently infected with HIV-1 or HBV that not only diminished recognition by epitope-specific CD8 T cells, but also inhibited recognition of wild-type epitope if both epitopes were present in the infected cell (Bertoletti et al., 1994b; Klenerman et al., 1994). The mechanism of
this phenomenon (TCR antagonism) is not completely understood, but is believed to involve delivery of an altered signal to the CD8 T cell by the mutated peptide/MHC class I complex, resulting in nonresponsiveness or diminished responsiveness to the target agonist peptide (Sette et al., 1996). Although TCR antagonism has only been demonstrated by using CTL clones, it has also been documented to occur when variant and wild-type epitopes are presented by different cells, a scenario that presumably mimics the situation in the infected host (Meier et al., 1995). Another mechanism similar to antagonism is interference with CTL priming (Plebanski et al., 1999). In this case, a variant epitope does not inhibit CTL effector function but, rather, the generation of activated antigen-specific CTLs from naive precursor CD8 T cells. This mechanism, identified in humans and mice infected with malaria, has not yet been described in any viral disease.

III. SELECTION OF CTL ESCAPE MUTANTS IN VIRAL ENCEPHALOMYELITIS

One of the best examples of a pathological setting in which CTL escape mutants contribute to virus persistence and the development of disease is that of mice infected with the neurotropic coronavirus, mouse hepatitis virus—strain JHM (MHV-JHM). Selection of escape mutants was not anticipated because immune selective pressure in the CNS, a site that is relatively protected from immune surveillance, might be predicted to be less than in the periphery.

Neurotropic coronaviruses, including MHV-JHM, the A59 strain of MHV (MHV-A59) and MHV-3, cause acute and chronic infections of the CNS in susceptible mice and rats (reviewed in Houtman and Fleming, 1996b; Lane and Buchmeier, 1997; Stohlman, Bergmann, and Perlman, 1998). MHV-JHM is highly neurovirulent and infection of susceptible mice results in widespread infection of neurons with a rapidly fatal outcome. However, more generally interesting are those model systems in which MHV-JHM and MHV-A59 are induced to cause either acute or chronic demyelination. General strategies for modifying the acute infection have been recently reviewed (Perlman, 1998; Stohlman, Bergmann, and Perlman, 1998), and two general approaches will be briefly described here. In the first, mice are infected with attenuated virus. Attenuated virus was cloned from pools of virus harvested from suckling mouse brain (Stohlman et al., 1982). Alternatively, viral mutants were selected by chemical mutagenesis or by treatment with neutralizing antibody directed against
the surface (S) glycoprotein (Dalziel et al., 1986; Fleming et al., 1986). In each case, the variant virus causes acute encephalitis in only a small percentage of mice but is able to persist and cause demyelination in most survivors. In mice infected with these variants, infectious virus is, in general, cleared by 2–3 weeks after inoculation. Clinical symptoms are most evident within the same time frame. Mice that survive the infection slowly recover, although evidence of ongoing demyelination can be detected for several months after the acute infection has resolved.

In the second approach, mice are infected with wild-type MHV-JHM and protected from the acute encephalitis by administration of anti-MHV antibody or MHV-specific CD4 or CD8 T cells (Buchmeier et al., 1984; Stohlman et al., 1986; Yamaguchi et al., 1991). This intervention also results in decreased infection of neurons, but does not prevent infection of cells in the white matter or demyelination. Virus clearance is enhanced by treatment with protective antibodies or T cells in some studies (Buchmeier et al., 1984; Yamaguchi et al., 1991), but not all (Stohlman et al., 1986).

In a variation of the latter approach, suckling C57BL/6 (B6) mice are protected from acute encephalitis with nursing by dams previously immunized to MHV-JHM (Perlman et al., 1987). The suckling mice are protected from acute encephalitis and remain asymptomatic for 3–8 weeks. At that time, a variable percentage (30–90%) develop histological evidence of demyelination and clinical signs of hind-limb paralysis. Immunization of the dams may be accomplished either by active immunization with infectious virus (Perlman et al., 1987), or by passive infusion of neutralizing antibody (Pewe, Xue, and Perlman, 1997), and with similar outcomes. The temporal course of this disease is very different from that observed in other models of MHV-JHM-induced demyelination, because mice are asymptomatic for several weeks before developing disease, and because infectious virus is not cleared. In a series of reports, Pewe et al. showed that escape from the cytotoxic CD8 T cell response was a major factor in the disease progression in these mice (Pewe et al., 1996; Pewe, Xue, and Perlman, 1997; Pewe, Xue, and Perlman, 1998). CTL escape mutations were detected in all mice that developed clinical disease several weeks postinfection in this model. In B6 mice, two CD8 T cell epitopes, encompassing residues 510–518 (S-510–518) (H-2Db-restricted) and 598–605 (S-598–605 (H-2Kb-restricted), of the S glycoprotein are recognized (Bergmann et al., 1996; Castro and Perlman, 1995). These two epitopes are located within a region of the protein that is prone to both mutation and deletion (termed the "hypervariable region") (Banner, Keck, and Lai, 1990;
Parker, Gallagher, and Buchmeier, 1989). The location of the epitopes within a region that can tolerate mutation, without loss of function, most likely contributes to the selection of CTL escape mutants. Mutations have only been detected in epitope S-510–518, the immunodominant epitope of the two (Castro and Perlman, 1996), and not in epitope S-598–605. Mutations are not detected in regions of the genome flanking epitope S-510–518 or in most MHV-specific CD4 T cell epitopes. The one possible exception is that a mutation is detected in a CD4 epitope encompassing residues 328–347 of the S glycoprotein (S-328–347) in several mice, although wild-type epitope is also detected in the same animals. The significance of this mutation is not known, because it does not appear to decrease recognition by CD4 T cells specific for the epitope (Xue and Perlman, 1997).

Mutations have been detected in amino acids at positions 2–7 of epitope S-510–518, with only a single variant generally being detected in any individual infected mouse (Pewe, Xue, and Perlman, 1997). A summary of mutations detected thus far is shown in Fig. 1. Based on the crystallographic structure of the H-2D\(^b\) molecule and on mutagenesis studies (Hudrisier et al., 1996; Young et al., 1994), some of these mutations are predicted to affect binding to the MHC class I molecule, whereas others inhibit binding to the TCR of the CD8 T lymphocyte. Mutations in epitope S-510–518 are selected within 10–12 days postinfection, a time at which virus is not cleared in other MHV infections (Houtman and Fleming, 1996a; Lin et al., 1999). Detection of CTL escape mutants at a time before virus clearance is expected to be complete is consistent with a role for these mutants in virus persistence. Notably, epitope S-510–518-specific CTL activity can be detected in the CNS as early as 5 days postinfection, prior to the detection of CTL escape mutants (unpublished observations). Mutations are also not detected in infected mice with severe combined immunodeficiency (SCID mice), suggesting that their selection is CD8 T cell-driven (Pewe, Xue, and Perlman, 1997). In this model, mice that do not develop hind-limb paralysis by 60–80 days postinfection remain asymptomatic. Mutations are only occasionally detected in asymptomatic mice at 60–80 days postinfection suggesting that escape from CD8 T cell surveillance correlates well with virus amplification in B6 mice but is not sufficient for the development of clinical disease.

As discussed above, the role of CTL escape mutants in viral pathogenesis is controversial. These mutants are not expected to be relevant to pathogenesis if the CTL response is directed at several CTL epitopes, as is believed to occur in many human infections. It has also been postulated that, even if a single epitope is immunodominant, a T cell
**CTL ESCAPE MUTANTS IN THE CNS**

Position within epitope:  

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
|*M* | M | M | T | M | T | M/T | M |

**Detected**  

- S  
- F  
- T  
- Y  
- F  
- D  
- R  
- L  

**Wild type epitope:**  

- C  
- S  
- L  
- W  
- N  
- G  
- P  
- H  
- L  

**Not detected:**  

- C  
- H  
- S  
- W  
- R  
- W  
- L  
- E  
- P  
- A  
- R

**FIG 1.** Summary of mutations detected in epitope S-510-518 in virus isolated from mice with hind-limb paralysis. A panel of peptides resulting from single nucleotide changes in the sequence of the wild-type epitope were tested in cytotoxicity assays. Only those changes resulting in a 20-fold decrement in activity are included in the figure. The amino acids listed above the wild-type sequence have been detected in virus isolates, whereas those listed below the wild-type sequence (underlined) have not yet been detected. The L to P change in position 3 results in only a 4-fold decrease in recognition but has been detected in a minority of cDNA clones in single mice with hind-limb paralysis (marked with parentheses). *MHC (M) or TCR (T) contact-based on published reports (Hudrisier et al., 1996; Hudrisier et al., 1995; Young et al., 1994).*

A response that is comprised of many CD8 T cell clonotypes would preclude CTL escape, because a single mutation would affect recognition by only a subset of the epitope-specific T cells (Ishikawa et al., 1998). To determine the biological significance of the mutations that were detected in MHV-infected mice, several additional experiments were performed. First, a panel of variant epitopes were analyzed in cytotoxicity assays, using either lymphocytes derived from the CNS of mice with acute encephalitis directly *ex vivo* or bulk populations of splenocytes cultured *in vitro* for 2 weeks with peptide S-510-518 (Pewe and Perlman, 1999; Pewe et al., 1996). This panel comprised all the peptides resulting from single nucleotide changes in epitope positions 2–7 and 9. These assays showed that nearly all of the mutations that were
selected in vivo resulted in a complete or substantial loss of recognition by either population of lymphocytes. Several additional mutations also resulted in a nearly complete loss of recognition by these lymphocytes, but have not yet been detected in vivo (Fig. 1) Strikingly, some mutations in position 9, a secondary anchor for binding to the MHC molecule, resulted in loss of recognition, but were never selected in infected mice. Whether this apparent selectivity reflects a sampling error, or is in fact biologically driven (i.e., results in detrimental changes in RNA or protein structure), remains to be determined.

The biological significance of these mutations was also addressed by infecting maternal antibody-protected suckling C57BL/6 mice with virus mutated in epitope S-510–518. Mice infected with this virus do not recognize epitope S-510–518 but still respond to epitope S-598–605. Variant virus-infected mice have significantly greater mortality and morbidity when compared to mice infected with wild-type virus (Pewe, Xue, and Perlman, 1998). These results suggest that the CD8 T cell response to epitope S-510–518 is an important component of the host response to MHV-JHM, and support the idea that escape from this response is beneficial to the virus and results in increased virus replication and disease progression.

These results suggest that the CTL response to epitope S-510–518 is monospecific, because single mutations in residues important for binding to either MHC antigen or the TCR result in substantial loss of activity in cytotoxicity assays. The monospecificity of the response is consistent with a monoclonal or an oligoclonal response to the epitope. To directly assess the diversity of the T cell response, TCR Vβ element usage and the complexity of the complementarity determining region 3 (CDR3) were determined. The TCR is a heterodimer consisting of an α and a β chain. The great diversity in the T cell response results from the large number of different V, D, and J elements in the germ line, coupled with imprecise joining at the V-D and D-J junctions (β chains) or V-J junction (α chain). The CDR3 encompasses the junctional region of the α and β chains and makes direct contact with the MHC/peptide complex. Analysis of a monoclonal or oligoclonal T cell response should reveal usage of only one or a small number of different Vβ and Vα elements and minimal heterogeneity of the CDR3. Sequence analyses of the CDR3 were facilitated by the use of soluble MHC/peptide tetramers specific for epitope S-510–518 (tetramer S-510). Soluble MHC class I/tetramers were first described by Altman et al., and have been shown, in several subsequent studies, to detect antigen-specific T cells with high sensitivity and specificity (e.g., Altman et al., 1996; Flynn et al., 1998; Murali-Krishna et al., 1998).
Epitope S-510–518-specific CD8 T cells were isolated directly from the CNS of mice with acute encephalitis by sorting with a fluorescent-activated cell sorter (FACS) after staining with anti-CD8 antibody and tetramer S-510. The tetramer S-510-positive T cells were analyzed initially for Vβ usage. The results showed usage of a large number of different Vβ elements by CD8 T cells responding to the epitope, with preferential usage of some Vβ elements occurring, specifically Vβ8 and Vβ13 (Pewe et al., 1999). Analysis of individual CDR3 sequences from a subpopulation of tetramer S-510-positive cells, those expressing the Vβ13 element, revealed substantial heterogeneity. The CDR3 sequences were shown to fit, approximately, a logarithmic distribution (Fisher, Corbet, and Williams, 1943; Pewe et al., 1999; Taylor, Kempston, and Woiwod, 1976). From this distribution, it was possible to calculate that approximately 300–500 epitope S-510–518-specific CD8 T cell clonotypes were present in the CNS of a mouse with acute encephalitis. Similar measurements using lymphocytes harvested from the CNS of mice with chronic demyelination revealed that approximately 100–900 different clonotypes were present in these animals. Previous studies suggested that either 3,000 (Butz and Bevan, 1998) or 600 (Bousso et al., 1998) precursor CD8 T cells for any single epitope were present in an individual mouse. Our calculation of the number of T cells recognizing epitope S-510–518 agrees more closely with the lower estimate.

Notably, none of the wild-type epitope S-510–518 RNA, or little of it, is present in the CNS of mice with chronic demyelination, but CD8 T cells specific for the epitope are still detectable. Whether this represents stimulation by residual wild-type antigen (which may be cleared more slowly than viral RNA [Knopf et al., 1998; Zhang et al., 1992], or by variant sequence remains to be determined. Furthermore, many of the β chain CDR3 sequences were detected in more than one mouse. In the case of mice with chronic demyelination, very few CDR3 sequences were unique to a single animal. Thus, unlike other experimental systems (Bousso et al., 1998), the repertoire of CD8 T cell TCRs recognizing epitope S-510–518 is not unique to each naive animal but is, in large part, common among all C57BL/6 mice. This lack of variability may facilitate the emergence of CTL escape mutants in a large fraction of infected mice.

These results suggest that CTL escape mutants are critical in virus persistence and in the development of clinical disease in mice infected with MHV-JHM. These results also show that CTL escape mutants are selected in the presence of a polyclonal, but monospecific, CD8 T cell response. As already mentioned, the importance of CTL escape
mutants in other infections is much less certain, suggesting that the following features, present in the MHV-JHM-infected CNS, must predispose to the selection of these mutants:

1. CTL escape mutants are observed in MHV-infected rodents only when B6 mice are inoculated at the suckling stage with virulent wild-type virus and protected from acute disease by nursing by immunized dams. Inoculation with virulent MHV-JHM and use of B6 mice are essential for disease to develop. Chronic demyelination does not develop if suckling mice are inoculated with the less neurovirulent A59 strain of MHV (unpublished observations). In most other models of MHV persistence, older mice are inoculated with attenuated strains. Persistence is manifested by the presence of viral RNA and ongoing demyelination in the absence of infectious virus (Adami et al., 1995; Houtman and Fleming, 1996a; Rowe et al., 1997). CTL escape mutants have not been detected in these models, possibly because clearance of infectious virus is so rapid (Bergmann et al., 1998). A normal immune system is also required, however, because even when clearance is delayed in mice infected with attenuated virus, as occurs in immunodeficient mice, CTL escape mutants are not commonly selected (Lin et al., 1999).

2. Infection with wild-type virus at the suckling stage (10-14 days old) is also crucial in this model. Three-week-old B6 mice inoculated with wild-type virus and protected from acute encephalitis by passive administration of small amounts of neutralizing antibody are protected from acute encephalitis, but they do not develop hind-limb paralysis at later times (Sun and Perlman, 1995). Demyelination can be detected in the spinal cords of these mice at 2 months postinfection. These mice have not formally been assessed for the presence of CTL escape mutations at late times after infection, but they clearly do not develop a clinical disease consistent with the selection of CTL escape mutants. These results suggest that inoculation of mice with virulent virus at the suckling stage is essential for the selection of CTL escape mutants or, as a minimum, for their clinical manifestation. This requirement for inoculation of suckling mice may reflect the immaturity of the immune system. A consequence of infecting mice at 10 days of life may be suboptimal clearance of virus. Consistent with this possibility, after inoculation at the suckling stage, low levels of infectious virus can be detected at 40 days postinfection in BALB/c mice (unpublished observations). BALB/c mice never develop evidence of hind-limb paralysis or other clinical disease at any time and develop completely normally (Castro et al., 1994; Perlman, Schelper, and Ries, 1987).

3. Another consequence of inoculation of mice with an immature immune system may be an aberrantly polarized immune response. A
striking feature of the immune response in most C57BL/6 mice infected with MHV at the suckling stage is the lack of an appreciable antibody response, when they are assayed by ELISA or in neutralizing assays (Jacobsen and Perlman, 1990; Perlman et al., 1987; Perlman, Schelper, and Ries, 1987). The CD4 and CD8 T cell response in maternal antibody-protected mice that develop chronic demyelination is proinflammatory and readily detected (Castro et al., 1994; Pewe et al., 1999). This strongly polarized, cell-mediated immune response in the presence of a defective humoral response may also predispose to the selection of CTL escape mutants. In support of this, BALB/c mice inoculated at the suckling stage in the maternal antibody model mount a significant antibody response and do not develop hind-limb paralysis (Castro et al., 1994; Perlman, Schelper, and Ries, 1987).

4. MHV infection of congenic B10.A(18R) (KbDdLd) mice results in the development of hind-limb paralysis, albeit at a lower frequency than in either B6 or C57BL/10 mice (Castro et al., 1994). Two CTL epitopes, S-598–605 and an Ld-restricted epitope encompassing residues 318–326 of the nucleocapsid (N) protein (N-318–326), are recognized in these mice. The immunodominant epitope recognized in B6 mice, epitope S-510–518, is not recognized in B10.A(18R) mice since these mice do not encode the H-2Db allele. Virus isolated from B10.A(18R) mice with hind-limb paralysis was evaluated for the presence of mutations in epitopes S-598–605 and N-318–326, and none were detected. The development of hind-limb paralysis in B10.A(18R) mice, in the absence of mutations in either CTL epitope, suggests that a factor expressed on the C57BL/6 background (decreased production of antibody?) may contribute to the increased amount of virus replication observed in these mice. The outgrowth of CTL escape mutants in B6 mice, in conjunction with this other putative factor, results in a much higher rate of clinical disease than is observed in B10.A(18R) mice in which CTL escape mutants are not selected.

5. A CTL response directed at a single immunodominant epitope enhances the likelihood that CTL escape mutants will be selected. In B6 mice, two MHV-specific epitopes are recognized. Cytotoxicity assays using CNS-derived lymphocytes from mice with acute encephalitis, directly ex vivo (Castro and Perlman, 1995), and limiting dilution assays using splenocytes harvested from mice intraperitoneally immunized with MHV (Castro and Perlman, 1996), suggested, in both cases, that the two epitopes were recognized by similar numbers of CD8 T cells. More recent measurements, using MHC class I peptide/tetramers to detect antigen-specific cells or methods to detect interferon-γ production after stimulation with peptide, confirmed
these results (unpublished observations). However, much more peptide S-598–605 is required to sensitize target cells for lysis. Thus, the CTL response in these mice may be functionally directed at a single epitope. Conversely, a CTL response to a single immunodominant epitope is not sufficient for CTL escape mutants to be selected. The CTL response in BALB/c mice is directed at a single epitope (N-318–326), yet these mice do not develop hind-limb paralysis in the maternal antibody-protection model (Castro et al., 1994), and CTL escape mutants are not selected (unpublished observations). The ability of the virus to tolerate mutations in the part of the protein that contains the target CD8 T cell epitope is also important. Epitope S-510–518 is in a region of the S protein prone to deletion and mutation, while the BALB/c epitope is located in a highly conserved region of the N protein. As mentioned above, mutations in this epitope are not detected in B10.A(18R) mice, even though the background of this strain may be favorable for the selection of CTL escape mutants.

6. MHV-JHM is neurotropic and the infection caused by this agent is confined, for the most part, to the CNS. As discussed above, the processes of initiation of an inflammatory response, trafficking to the site of inflammation, and antigen presentation within the CNS differ in several aspects from similar processes occurring at extraneural sites. In particular, MHC class I expression is minimal in the normal CNS, and while clearly upregulated within the infected CNS, determining the cellular sites of expression remains an area of active research. Therefore, while it is speculative at present, it is formally possible that infection within the CNS results in a modified immune response that increases the likelihood that CTL escape mutants will be selected during the course of an infection.

IV. CONCLUSIONS AND FUTURE DIRECTIONS.

What can be concluded from these studies about the role of CTL escape mutants in the pathogenesis of viral infections in the CNS? The following conclusions may also be applicable for CTL escape mutants arising in extraneural tissue.

First, CTL escape mutants are not commonly selected in most infections. One explanation for this is that the selection of CTL escape mutants is uncommon if the CD8 T cell response is directed against several epitopes. However, a CD8 T cell response directed against one epitope or a few, has been observed in several human and experimental infections (Cole, Hogg, and Woodland, 1994; Flynn et al., 1998;
Lehner et al., 1995; Moss et al., 1991; Murali-Krishna et al., 1998; Wallace et al., 1999)—suggesting that an immunodominant response is not unusual. Nevertheless, CTL escape mutants are not detected in most experimental settings even when only one epitope is immunodominant, or a few are.

Second, the recognition of an epitope by a diverse population of CD8 T cell clonotypes does not prevent emergence of these variants. What does appear to be true, however, is that the diversity of the response matters less than whether the different CD8 T cell clonotypes recognize the same or different parts of the MHC/peptide complex. A response narrowly focused on one part of the complex will facilitate the selection of CTL escape mutants. The CTL response to epitope S-510–518 is very sensitive to changes in residues 4 and 6 (Pewe and Perlman, 1999; Pewe et al., 1996), two residues important for binding to the TCR (Young et al., 1994). Strongly focused recognition of central residues of an immunodominant H-2Kb-restricted epitope, which is recognized in Sendai virus-infected B6 mice, has also been reported (Cole, Hogg, and Woodland, 1995).

Third, in mice infected with MHV and in some humans persistently infected with HIV-1, HBV, or hepatitis C virus, CTL escape mutants play an important role in virus amplification and disease progression. In most cases, however, it is likely that there would not have been sufficient time for selection of CTL escape mutants if virus clearance were rapid and complete. Thus, selection of CTL escape mutants in the absence of any other factor is probably not sufficient to prevent rapid virus clearance from an infected host. In MHV-infected B6 mice, their selection appears to be facilitated by a suboptimal humoral immune response. This deficient antibody response may be a consequence of infection of suckling mice in the presence of maternally derived antibody. Maternal immunization has been shown to impair immune responses in other infections (Wang et al., 1998), although it has never been shown to affect selectively humoral, and not cellular, immunity.

Fourth, an interesting aspect of this model system is the number of questions that it raises about the dynamics of MHV growth and cellular tropism in the CNS. Unlike the situation in mice chronically infected with TMEV, in which macrophages are the main reservoir for virus (Lipton, Twaddle, and Jelachich, 1995), no single type of CNS cell has been identified as the predominant target in mice persistently infected with MHV. The selection of CTL escape mutants in the CNS of MHV-infected mice demonstrates that MHV infection of a cell expressing MHC class I antigen is a critical part of the pathogenic process. Astrocytes, macrophages/microglia, and oligodendrocytes are all
infected in these animals (Perlman and Ries, 1987; Xue et al., 1999). Of all of these cells, only macrophages/microglia express detectable levels of MHC class I antigen in MHV-infected mice with chronic demyelination (Xue et al., 1999). One interpretation of these results is that macrophages/microglia are in fact the primary site of productive virus infection, with replication in oligodendrocytes or astrocytes being of secondary importance or abortive. Infection of the latter two cells might be crucial for the development of clinical disease, but might be less important for propagation of the virus. Alternatively, astrocytes or oligodendrocytes might express MHC class I antigen, albeit at levels below the detection limits of assays presently available. Escape from CD8 T cell surveillance might occur in these cells, thereby resulting, simultaneously, in increased virus replication and disease progression. Infection with MHV affects MHC class I antigen expression by astrocytes in vitro and in newborn mice but this has not been demonstrated in vivo in older mice (Correale et al., 1995; Gilmore, Correale, and Weiner, 1994; Suzumura et al., 1988; Suzumura et al., 1986). Conversely, proof that astrocytes or oligodendrocytes were the most important sites of MHV-JHM replication and, therefore, the sites where selection of CTL escape mutants occurred, would provide evidence that these cells express MHC class I antigen.

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