EVASION OF THE HUMAN CELLULAR IMMUNE RESPONSE BY HERPES SIMPLEX VIRUS

by

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ABSTRACT

Herpes simplex virus (HSV) is unusual in its ability to cause recurrent infection in a host with an ostensibly competent immune system. This thesis describes two mechanisms by which HSV evades the cellular immune response, which contribute to its ability to persist.

Human cells infected with HSV inhibit lysis by natural killer (NK) and lymphokine-activated killer (LAK) cells. Contrary to the conclusions of previous workers, this effect is due to infection of the lytic effector cells, as shown by experiments with mutant HSV and by immunofluorescence staining of effector cells in contact with HSV-infected targets. The inhibition of lytic effector cells by HSV by cell-to-cell spread may play an important role late in the infectious cycle.

Early in the infectious cycle, infectious virus is not present and the above mechanism cannot take effect. Instead, HSV imposes a block on the cellular pathway which presents antigen to $CD8^+$ cytotoxic T lymphocytes. The result is that HSV-infected human fibroblasts show abnormalities of their class I major histocompatibility complex (MHC class I) similar to those seen in antigen-presenting mutant cell lines: the MHC class I is retained within the ER and is misfolded and unstable, implying that peptide is not associated with the heavy chain/β_2-microglobulin complex. Experiments with mutant and recombinant viruses established that this effect is due to an HSV immediate-early protein, ICP47, and showed that cells expressing ICP47 are not efficiently recognized and lysed by CD8+ CTL. Since ICP47 is not detectably membrane-associated, it presumably affects some cytoplasmic component of the antigen-processing pathway. One candidate for this cellular target is a small (8.5 - 9 kDa) protein, which, like ICP47, is located within the cytoplasm and nucleus of certain cell types. This protein was detected by its ability to bind to a protein consisting of ICP47 fused to the carboxy terminus of glutathione S-transferase (GST).

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PREFACE

The data in this thesis are presented in three chapters, two of which have been published in peer-reviewed journals. *Chapter 1* ("Direct contact with herpes simplex virus-infected cells results in inhibition of lymphokine-activated killer cells because of cell-to-cell spread of virus") was published in The Journal of Infectious Diseases (168:1127-1132, 1992). This paper demonstrates that the inhibition of lytic function seen when lymphokine-activated killer cells are incubated with HSV-infected cells is due to infection of the LAK cells. *Chapter 2* ("A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ lymphocytes.") was published in Cell (77:525-535, 1994) and shows that the HSV immediate-early protein ICP47 blocks antigen processing pathways in human fibroblasts. Chapters 1 and 2 are both followed by brief discussions, which describe related work which appeared in the literature after these papers were accepted for publication and which incorporate the particular findings into the broader field of HSV immune evasion. *Chapter 3* ("Herpes simplex virus ICP47 binds to an 8.5 kDa cellular protein.") describes preliminary findings in the search for the mechanism of action of ICP47. These chapters are preceded by an Introduction to viral immune evasion in general and the role of the immune system in herpes simplex virus infections in particular. The chapters are followed by a Discussion, which unifies the concepts and issues raised in the thesis and which considers applications and further research.

The thesis candidate wrote the initial drafts of the scientific manuscripts and subsequent drafts were revised in collaboration with my thesis supervisor, Dr. D.C. Johnson. Dr. Johnson also performed most of the immunofluorescence experiments described in Chapter 2. Other coauthors were included for Chapter

2. Ms. Cindy Roop, in collaboration with the thesis candidate, performed endoglycosidase H assays with the wild-type and mutant HSV (Figure 2A - C; Figure 4). Dr. David W. Andrews was primarily responsible for performing the membrane-binding assay (Figure 8). Dr. Stanley R. Riddell provided reagents for the human cytomegalovirus CTL assays and Dr. F.L. Graham assisted in the rescue of the adenovirus vectors. John Rudy also assisted in the rescue of the adenovirus vectors. Dr David Johnson performed most of the immunofluorescence experiments (Figure 7).

TABLE OF CONTENTS

INTRODUCTION

One of the major hurdles a virus must surmount is the host immune system. Many, if not all, viruses have developed ways of coping with host immunity. In most cases, the virus can only temporarily avoid the effects of the immune system; the result is a brief infection, limited by the mature immune response, as with classic rhinovirus infections. In other cases, viruses may be able to more drastically alter the balance; the ability of adenoviruses to establish long-lasting infections is probably at least partly related to their arsenal of immune evasion gene products.

The Herpesviridae are relatively large viruses with the characteristic ability to persist in the infected host for long periods in a latent form. Not surprisingly, members of this family have evolved very sophisticated methods of dealing with the immune system. This thesis will describe two methods used by herpes simplex virus: the ability to block lysis by natural killer (NK) and lymphokine-activated killer (LAK) cells (Chapter 1), and the ability to prevent recognition by $CD8^+$ cytotoxic T lymphocytes (CTL) (Chapters 2 and 3).

Natural killer and lymphokine-activated killer cells

NK cells (reviewed in Kärre, 1993) are lymphocytes with the ability to lyse a relatively broad range of targets. This definition is based on function, rather than phenotype; however, most NK cells are large granular lymphocytes expressing the surface markers CD16 and CD56, and lacking specific T and B cell markers. LAK cells, which are functionally similar to NK cells (although LAK are capable of lysing a wider range of targets, and are more efficient in their lysis) are derived from NK cells, or, less often, from $CD8^+$ or $CD4^+$ lymphocytes, by

treatment with a number of cytokines, particularly IL-2 (Grimm et al., 1982). NK cells compose about 5 - 15% of circulating lymphocytes in humans, and are important mediators of anti-viral immunity, particularly in the very early stages of an infection.

Target recognition by NK and LAK cells is not well understood. NK cell lysis is strongly correlated with low expression of the class I major histocompatibility complex (MHC class I) on target surfaces (reviewed in Ljunggren and Kärre, 1990; Kaufman et al., 1992; Kaufman et al., 1993), suggesting that surface MHC class I may deliver an inhibitory signal to NK cells. However, it is clear that factors other than MHC class I expression must also play a role in NK and LAK recognition, as in some cases lysis does not correlate with target cell MHC class I expression (Sarin et al., 1993; Mason et al., 1993; Malnati et al., 1993; Kesari and Geliebter, 1993). A number of cell surface proteins which may be responsible for recognition or inhibition have been identified (Giorda et al., 1990; Moretta et al., 1990; Moretta et al., 1993; Harris et al., 1993; reviewed in Hofer et al., 1992), but their effects and relative importance are not yet clear.

$CD8^+$ T cells and antigen processing

The cell-mediated immune response includes cytotoxic T lymphocytes (CTL), which respond to viral infection by specifically lysing infected cells. CTL generally express the CD8 accessory molecule, although some CTL express instead CD4. (CD4 and CD8 are mutually exclusive in mature T lymphocytes.) $CD8^+$ CTL interact with MHC class I, which is expressed on the surface of most cells. The interaction is mediated by both the CD8 molecule, which binds to conserved regions of the polymorphic MHC class I complex, and by the T cell

receptor, which binds to variable regions. The result of specific recognition by the TcR/CD8 complex is activation of the CTL, the delivery of toxic signals, and ultimately lysis of the target cell (reviewed in O'Rourke et al., 1993; O'Rourke and Mescher, 1993).

The MHC class I complex is a trimolecular complex, consisting of a heavy chain, a light chain ($ß_2$-microglobulin: $ß_2$-m), and a short peptide, generally 8 or 9 amino acids long. The combination of peptide and surrounding heavy chain residues is the aspect of the MHC class I which is recognized by the TcR, and therefore the peptide is responsible for conferring the specificity of recognition. The peptide is derived from proteolytic degradation of cytoplasmic proteins. The machinery involved in this proteolysis includes, and may be limited to, the proteasome complex. The proteasome is a large complex, composed of some 15 - 25 proteins of 20-35 kDa each, which is capable of a wide range of proteolytic activites (reviewed in Rivett, 1993), Proteins are tagged for degradation by the proteasome by covalent attachment of an 8.5 kDa protein, ubiquitin (Matthews et al., 1989; Kanayama et al., 1992; Seufert and Jentsch, 1992; reviewed in Finley, 1991; Hershko and Ciechanover, 1992; Jentsch, 1992). As the major cytoplasmic protease activity, proteasomes are obvious candidates for a role in antigen processing. The association with antigen processing was made more plausible by the discovery that two proteins encoded in the MHC class II region, LMP-2 and LMP-7, are components of a subset of proteasomes (reviewed in Goldberg and Rock, 1992; Brown et al., 1993; Frentzel et al., 1993). Although no convincing role for LMP-2 and LMP-7 in antigen processing has been demonstrated (Momburg et al., 1992; Arnold et al., 1992; Yewdell et al., 1994), the recent findings that ubiquitin is required for the presentation of at least one epitope

(Michalek et al., 1993), and that the proteasome can generate appropriate peptide epitopes (Dick et al., 1994) clearly suggest a role for the proteasome. It is also possible, however, that other cytoplasmic proteases can also be involved in antigen processing (Eisenlohr et al., 1992).

After proteolysis, the peptide is transferred into the ER by the TAP1/TAP2 complex; these proteins are integral membrane proteins, members of the ATP-binding cassette family of transport molecules (Neefjes et al., 1993; Androlewicz et al., 1993). There is circumstantial evidence that heat-shock proteins (HSP) may be involved in transport to and from the TAP complex (Srivastava et al., 1994). Cytoplasmic (Udono and Srivastava, 1993) and ER (Li and Srivastava, 1993). HSP (HSP70 and grp94, respectively) are associated with peptides, which are capable of inducing specific cell-mediated immunity. The observation that ATP causes the release of peptides from HSP70 (Udono and Srivastava, 1993), coupled with the finding that there is an ATP-dependent step in MHC class I assembly within the ER (Lévy et al., 1991) suggests that HSP may specifically bind and transport peptides from the proteasome to the TAP complex, and from the TAP complex to the nascent MHC class I complex. Some further support for this idea may come from the observation that several HSP are encoded within the MHC class II region. However, definitive evidence for this is still lacking, and the recent finding that the TAP transporters and MHC class I are associated (Ortman et al., 1994; Suh et al., 1994) makes such a function less likely within the ER. Further, if the ER HSP are not involved, then the binding of antigenic peptide to the HSP which was the major evidence for HSP's role in antigen processing may well be unrelated to antigen processing, so this observation also weakens the case for the cytoplasmic HSP.

Within the ER, the peptide interacts with the immature complex of heavy chain and β_2-m. Peptides do not bind to MHC class I at random: a few residues in the 8 or 9 amino acid-long peptides act as anchors for binding to the MHC class I, and these anchor residues differ for each MHC class I haplotype (Falk et al., 1991; reviewed in Falk and Rotzschke, 1993) The formation of the trimolecular complex results in a conformational change in the complex, as measured by the ability to react with monoclonal antibodies; the trimolecular complex is also considerably more stable than is the complex of heavy chain/β_2-m. The conformational change induced by peptide binding may release the complex from an association with calnexin (Jackson et al., 1994) or with the TAP transporters (Ortmann et al., 1994; Suh et al., 1994) thereby allowing egress from the ER and cell-surface expression.

Viruses and immune evasion

Several recent reviews (Wold and Gooding, 1991; Gooding, 1992; McFadden and Kane, 1993; Smith, 1993) discuss viral immune evasion; a brief summary is included here.

In general, persistent viruses (particularly adenoviruses and herpesviruses) have the widest range of immune-evasion strategies. The major exceptions to this rule are members of the poxvirus family, which, with their large genome, can afford to include a number of non-essential, virulence-enhancing genes. Included among these genes are a number which exhibit significant homology to mammalian genes; the implication is that the viruses have incorporated cellular genes during their evolution. Several are homologous to cytokines or to cytokine receptors. The T2 open reading frame of Shope fibroma virus (Smith et al., 1990) and myxoma virus (Upton et al., 1991) encode secreted proteins with

homology to the tumour necrosis factor (TNF) receptor, and the M-T7 ORF of myxoma virus encodes a secreted homologue of the interferon-γ (IFNγ) receptor (Upton et al., 1992). These proteins enhance viral virulence, presumably by blocking the antiviral effects of TNF and IFN-γ (Upton et al., 1991; Upton et al., 1992) . As well, there is evidence that poxviruses also interfere with cell-mediated immunity, although the viral proteins and the mechanisms involved have not been identified. Late in infection with vaccinia virus, cells are inefficiently recognized by CTL, implying that vaccinia imposes a block on antigen presentation (Townsend et al. 1988). In addition, cells infected with myxoma virus show a dramatic reduction in cell-surface MHC class I. The latter is not due to inhibition of host protein synthesis, as the level of expression drops more rapidly in infected cells than in cells treated with cycloheximide (Boshkov et al., 1992).

Adenoviruses include some of the best-studied examples of viral immune evasion. Most adenoviruses (with the exception of the group A adenoviruses) express a 19 kDa glycoprotein (E3 gp19K) which binds to MHC class I within the endoplasmic reticulum (ER) and prevents egress from the ER (Burgert and Kvist, 1985; Andersson et al. 1985). *In vitro,* the effect of overexpression of E3 gp19K is to reduce lysis of targets by CTL (Gooding and Wold, 1990), although there seems to be little protective effect in cells infected with adenoviruses (Routes and Cook, 1990) unless there is prior expression of the E1a protein (Routes et al., 1993). Adenovirus serotype 12, a member of Group A, also downregulates MHC class I expression, but at the level of transcription (Schrier et al., 1983).

Adenoviruses are also able to evade the effects of some cytokines. Tumour necrosis factor (TNF) is able to cause lysis of many virus-infected or

tumour cells by a mechanism which resembles apoptosis. Cells infected with adenoviruses are resistant to this effect (Gooding et al., 1988). The resistance to TNF lysis seems to involve multiple proteins, depending on the cell type and species. The first protein identified as having a role in this protection was an E3 protein of 14.7 kDa (Gooding et al., 1988). As well, a complex of the 10.4 and 14.5 kDa E3 proteins (Gooding et al., 1991b), and the E1b 19 kDa protein (Hashimoto et al., 1991; Gooding et al., 1991a), are also capable of protecting certain cells against TNF-mediated lysis. Since the E1b 19 kDa protein has a more general effect in blocking apoptosis (Lowe and Ruley, 1993; Debbas and White, 1993), and since other effects of TNF (e.g. upregulation of MHC expression) are not generally blocked, the anti-TNF effect may be just one aspect of a more general resistance to apoptosis. Adenoviruses are also resistant to the antiviral effects of IFNγ, which can cause a general blockade of protein synthesis. This effect is mediated by activation of the double-stranded RNA-dependent protein kinase DAI, by binding to double-stranded RNA. Adenoviruses express small RNAs (VA RNAs) which bind to DAI and block its effect (O'Malley et al., 1986; Kitajewski et al., 1986), allowing the adenoviruses to use the protein synthesis machinery of the host cell even in the presence of IFNγ.

Epstein-Barr virus (EBV) encodes a homologue of the cytokine IL-10 (Hsu et al., 1990). Both human and viral IL-10 alter the immune response in a number of ways; in particular, monocyte function is affected, leading to a reduction in antigen-specific T cell proliferation (Moore et al., 1993; de Waal Malefyt et al., 1993), an effect which could obviously reduce antiviral immune responses.

Cells infected with either human (Barnes and Grundy, 1992) or murine cytomegalovirus (CMV) (Campbell et al., 1992) express lower amounts of cell-

surface MHC class I than do uninfected cells. The cause of this reduced expression is not clear, and appears to differ between HCMV and MCMV. In the very early stages of MCMV infection, MHC class I is retained within the ER or *cis*-Golgi (del Val et al., 1989), although the previous steps in antigen processing appear to be functional in that peptide has been added to the MHC class I complex (del Val et al., 1992). In HCMV infection, it is not clear whether a similar effect occurs; rather, there is a dramatic degradation of the MHC class I heavy chain in the ER or Golgi (Beersma et al., 1993).

Herpes simplex virus

Herpes simplex virus (HSV) is a double-stranded DNA virus with a genome of about 150 kilobase pairs (kb), encoding some 76 genes. The genome is organized into long and short unique regions (U_L and U_S) separated by repeated regions. HSV proteins are expressed in a temporally organized fashion, with immediate-early (IE) genes being expressed immediately following infection of cells; some IE proteins induce the expression of early (E) genes, which are generally involved in DNA replication. Following DNA replication, expression of late (L) genes (generally structural viral components) is induced. There is considerable overlap between the categories, particularly with regard to E and L genes. Virion structural components, delivered into the cells on infection, are the first viral proteins to affect the cell; these include the virion host shutoff protein (Vhs) and the transactivator of the IE genes, Vmw65 (reviewed in Roizman and Sears, 1990; Hay and Ruyechen, 1992).

HSV infection has a profound effect on cellular function. Cellular protein synthesis is rapidly inhibited, various gene products are abnormally induced (e.g.

Alu elements), and a wide range of cytopathic effects are seen. Cells are generally lysed within 24 to 48 hours of infection. An exception to this rule is infected neurons; *in vivo*, in appropriate conditions, HSV enters a latent phase in these cells, in which a very limited subset of gene products are expressed (Stevens, 1989).

HSV is a very common human parasite. Most infections are asymptomatic, with the virus replicating briefly in superficial tissues, spreading up innervating axons, and establishing a latent infection in sensory ganglia. The most common sites of infection are the mouth (HSV type 1) and the genitalia (HSV type 2) (Aurelian, 1990; Mertz, 1990; reviewed in Whitley, 1990), with latent infection in the trigeminal or dorsal root ganglia respectively. It has also been suggested that HSV can establish latent, or persistent, infection in bone marrow and blood (Cantin et al., 1994).

In some cases infection results in disease, ranging from mild superficial lesions to severe encephalitis. It is generally accepted that stress or exposure to UV light can induce reactivation from latent infection, with the most common manifestation being the notorious cold sores or genital lesions. Acute infection can result in ocular disease (herpetic keratitis or keratoconjunctivitis), a major cause of blindness in North America. Herpetic encephalitis is a rare, but very serious, consequence of infection; it is most common in newborns, particularly those born to mothers experiencing primary HSV-2 genital disease (Arvin, 1991). Herpetic encephalitis has a mortality rate of about 80% and survivors frequently show neurological complications (Whitley, 1990).

Other HSV diseases include infection of the skin, the respiratory tract (rarely), and, in immune compromised individuals, infections of the central nervous system, the respiratory tract, or the gastrointestinal system (Whitley, 1990).

Immunity to HSV

Most studies on HSV immunity have used the mouse as a model. HSV infection in mice causes a wide range of symptoms, ranging from no disease to fatal acute encephalitis, depending on the route of administration, the virus dose and strain, and the strain of mouse (Kastrukoff et al., 1986). Since similar studies have not, of course, been carried out in humans, it is not clear how different the disease in mice is from that in humans. It is likely that there are at least some qualitative differences; for one thing, latency in mice does not normally result in spontaneous recurrences as it does in humans (reviewed in Roizman and Sears, 1990). Conclusions based on mouse studies must therefore be treated with caution. On the other hand, human studies may also be difficult to interpret. Most have involved relatively few cases, and represent only a snapshot in the course of a lifelong infection. As well, control groups are rarely used, probably in part because of the difficulty of finding humans who have not been exposed to HSV. Instead, individuals may be tested before and after clinical recurrence of a latent infection: yet the time immediately after recurrence is likely to represent the most rapidly changing period of a remarkably dynamic host-parasite relationship.

HSV infection can probably be prevented by an adequate antibody response, whether to the whole virus or to various components (reviewed in Corey and Spear, 1986). Presumably in this situation extracellular virus can be rapidly neutralized. However, after the first cell is infected, antibody may be less

important for clearance of infection. HSV is a highly cell-associated virus, which can spread from cell to cell without ever entering the extracellular mileau, and therefore the opportunities for antibody-mediated clearance may be limited. In fact, HSV reactivation can occur in the presence of high titres of neutralizing antibody (Douglas and Couch, 1970; Zweerink and Stanton, 1981; Vestey et al., 1990; Kohl, 1992).

Non-specific immune cells, particularly macrophages and NK cells, are the first to be involved in immune clearance of HSV. These probably limit replication, thus reducing the effective infectious dose of virus while the specific immune response has a chance to develop (reviewed in Domke-Opitz and Sawatzkym, 1990). This is supported by experiments showing that transfer of non-immune spleen cells, particularly those treated with poly-I:C, to susceptible mice reduced HSV titres in recipients, an effect mediated by cells which were of the NK phenotype (Rager-Zisman et al., 1987). Similarly, in vivo depletion of NK cells makes mice more susceptible to HSV infection (Habu et al., 1984), although this may be mediated by IFNγ (Bukowski and Welsh, 1986). A single example exists of a human who lacked NK cells, with no other apparent immune defects; she had recurrent herpesvirus (CMV, VZV, and HSV) infections (Biron et al., 1989). In vitro, NK cells are capable of lysing HSV-infected cells (Colmenares and Lopez, 1986; Oh et al., 1990; Fitzgerald-Bocarsley et al, 1991).

Experiments involving adoptive lymphocytes or selective depletion of various lymphocyte classes (Nash et al., 1987) show that non-specific immunity is insufficient to prevent infection altogether. In both mice and humans, specific cell-mediated immunity is most important in limiting HSV spread (Corey and Spear, 1986). In mice, targeted deletion of specific cell types (Nash et al., 1981;

1987) and adoptive transfer experiments (Nash et al., 1980; Larsen et al., 1983; Bonneau and Jennings, 1989) suggest that both $CD8^+$ and $CD4^+$ lymphocytes are involved in protection, with neither being completely effective alone.

In humans, anti-HSV CTL are predominantly $CD4^+$, while $CD8^+$ HSV-specific lymphocytes are relatively rare (Yasukawa and Zarling, 1984a; Yasukawa and Zarling, 1984b; Schmid, 1988; reviewed in Schmid and Rouse, 1992). In mice, the reverse is true: the majority of anti-HSV CTL are $CD8^+$, with occasional $CD4^+$ CTL being detected (Martin and Rouse, 1990).

Immune evasion by herpes simplex virus

HSV is capable of replicating both in tissue culture and in vivo in the presence of high titres of neutralizing antibodies. A major reason for this ability is the virus' ability to spread directly from cell to cell, so that it does not come into direct contact with antibody. As well, HSV encodes receptors for complement and Fc, which may protect virus-infected cells and the virus itself from antibody-mediated cytolysis (reviewed in York and Johnson, 1994). The glycoprotein gC binds to the C3b component of complement, and is capable of reducing complement-mediated cell lysis (Friedman et al. 1984; McNearney et al., 1987; Frank and Friedman, 1989; Harris et al., 1990). The complex of gE and gI binds to the Fc region of antibodies (Johnson and Feenstra, 1987; Johnson et al., 1988; Hanke et al., 1990), inducing bipolar bridging of antibodies on the surface of infected cells (Frank and Friedman, 1989; Van Vleit et al., 1992), reducing complement-mediated lysis of infected cells and reducing antibody-dependent cell-mediated cytotoxicity (ADCC) (Dubin et al., 1991).

NK cell activity appears to drop during, or immediately before, HSV reactivation (Rola-Pleszczynski and Lieu, 1984; Kuo and Lin, 1990). It is not clear, however, whether this is a cause or an effect of HSV reactivation. In vitro, NK and LAK cells in contact with HSV-infected targets are rendered unable to lyse normally sensitive targets (Confer et al., 1990); this phenomenon is discussed further below (Chapter 2). Similarly, CD8+ CTL are also rendered inactive following contact with HSV-infected cells (Posavad and Rosenthal, 1992), apparently due to infection of the CTL (Posavad et al., 1993; Posavad et al., 1994), and monocytes infected with HSV are defective in several functions (Hayward et al., 1993). These effects, if they occur in vivo as well as in vitro, may play a role in the mild immune suppression observed during HSV infection (Sheridan et al., 1982; Rola-Pleszczynski and Lieu, 1984; Vestey et al., 1989; Rinaldo, 1990).

Although human CD8+ lymphocytes from HSV-seropositive donors can lyse HSV-infected syngeneic lymphoblastoid cell lines (LCL) (Yasukawa et al., 1989; Posavad and Rosenthal, 1992; Posavad et al., 1993) and some other cell types (Torpey et al., 1989; Cunningham and Noble, 1989), several studies have found that CD8+ CTL (either bulk cultures or clones) are not capable of lysing syngeneic fibroblasts (Posavad and Rosenthal, 1992; Koelle et al., 1993). It is worth noting, however, that other workers have succeeded in lysing HSV-infected fibroblasts with human HSV-specific syngeneic CTL (Sethi et al., 1980). This may be related in part to the observation that both HSV-1 and HSV-2 reduce the surface expression of MHC-I relatively late in the infectious cycle. HSV-2 is more effective than HSV-1 in reducing cell-surface MHC class I (Carter et al., 1984). Although the virion host shutoff function, being more aggressive in HSV-

2 than in HSV-1 (Fenwick and Everett, 1990), may play a part in the differential downregulation of surface MHC class I, the use of intertypic recombinants has mapped the region responsible to between 0.82 to 1.00 map units of the HSV-2 genome (Jennings et al., 1985), and therefore ICP47 may be involved (see Chapter 2).

CHAPTER 1

Direct Contact with Herpes Simplex Virus–Infected Cells Results in Inhibition of Lymphokine-Activated Killer Cells because of Cell-to-Cell Spread of Virus

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Natural killer (NK) and lymphokine-activated killer (LAK) cells are disarmed after contact with herpes simplex virus (HSV)–infected cells. Cells infected with HSV-1 mutants that lack glycoproteins essential for viral entry into cells (gB, gD, gK, gH, and gL) did not inhibit LAK cells; cells infected with HSV-1 mutants that lack glycoproteins not required for virus entry into cells (gE, gI, gG, and gJ) inhibited lysis. LAK cells became infected after contact with target cells infected with wild-type HSV-1 but not with a gD⁻ HSV-1, which cannot spread from cell to cell. Because LAK cells were inhibited only by very high concentrations of cell-free preparations of HSV and because neutralizing antibodies did not prevent infection of LAK cells in contact with infected cells, infection of LAK cells is probably greatly enhanced by the apposition of the effector and target cell membranes during target recognition. Disarming of immune effector cells by infection may be a general strategy for immune evasion by HSV.

Herpes simplex virus (HSV) initially infects mucous membranes, then follows innervating axons to sensory ganglia where it establishes a lifelong latent infection interspersed with bouts of recrudescence. Although the control of HSV infection is poorly understood, it is believed that cell-mediated immunity is important for preventing and limiting recurrent disease [1]. During periods of recrudescent disease, parameters of cell-mediated immunity seem to be somewhat depressed [2–4], although it is not known whether this is a cause or an effect of reactivation.

Natural killer (NK) cells are lymphocytes capable of lysing a broad range of abnormal targets (e.g., virus-infected or tumor cells) in a non–major histocompatibility class–restricted manner. Lymphokine-activated killer (LAK) cells are produced by exposing T lymphocytes or NK cells to high concentrations of certain cytokines; LAK cells have similar functions to NK cells but can lyse a wider range of targets than can NK cells. Although a number of candidates for NK cell receptors have been proposed, it is not yet clear how NK or LAK cells recognize their targets. Recently, Confer et al. [5] demonstrated that HSV-infected human endothelial cells or human fibroblasts were resistant to NK- or LAK-mediated lysis late (but not early) in the course of the infection. Furthermore, after exposure to infected cells, NK and LAK cells were unable to lyse normally susceptible targets. This effect was abrogated by treatment of the infected endothelial cells with tunicamycin, a drug that impairs glycosylation. Since direct infection of NK or LAK cells with HSV did not inhibit lysis, it was concluded that HSV-infected cells may express a glycoprotein capable of inhibiting NK and LAK function.

HSV-1 encodes at least 10 glycoproteins, of which 5 (gB, gD, gH, gK, and gL) are required for the production of virus capable of infecting cultured cells, while 5 (gC, gE, gG, gI, and gJ) are not required for growth in cultured cells (reviewed in [6, 7]). We used a panel of HSV-1 mutants unable to express individual glycoproteins to determine which were necessary for LAK inhibition. We investigated whether a single HSV glycoprotein mediated this effect and whether LAK cells became infected after direct contact with HSV-infected cells, thus leading to inhibition of LAK lytic function.

Materials and Methods

Cells and viruses. The viruses used were the wild-type HSV-1 strains KOS [8], F [9], and mP [10] and the HSV-1 mutants KO82 (gB⁻) [11]; MP4 (gC⁻) [10]; FUS6KAN (gD⁻) [12]; FgDβ (gD⁻ and gI⁻) [13]; FgEβ (gE⁻), where the US8 gene of HSV-1 (F) was disrupted by a *lacZ* gene cassette (unpublished data); In1404 (gE⁻) [14], US5::Tn*5* (gJ⁻) [15]; SCgHZ (gH⁻) [16]; FUS7KAN (gI⁻) [14]; US4::Tn*5* (gG⁻) [15]; FgKβ (gK⁻), where the UL53 gene was disrupted by a *lacZ* gene cassette (unpublished data); and KOSgLβ (gL⁻) [7]. MP4, FgEβ, US5::Tn*5*, FUS7KAN, and US4::Tn*5* were propagated and titered on Vero cells; complementing cell lines were used to propagate and titer KO82 (VB38 cells) [11], FUS6KAN and FgDβ (VD60 cells) [13], SCgHZ (F6 cells) [16], FgKβ (VK302 cells), and KOSgLβ (VL303 cells) [7]. Vero, VB38, and F6 cells and normal human fibroblasts (10–18 passages after being established from a human skin biopsy) were cultivated in α-MEM supplemented with glutamine, penicillin, streptomycin, and 10% fetal calf serum. K562 cells were cultivated in RPMI 1640 medium with the same supplements. VD60, VK302, and VL303 cells were cultivated in Dulbecco's MEM lacking histi-

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dine and supplemented with histidinol, glutamine, penicillin, streptomycin, and 10% fetal calf serum.

Lysis assays. Peripheral blood lymphocytes were derived by Ficoll-Paque (Pharmacia Biotechnology, Uppsala, Sweden) separation of blood from normal human volunteers. Lymphocytes (2×10^6 cells/mL) were incubated in RPMI 1640 supplemented with 10% fetal calf serum, glutamine, penicillin, streptomycin, and 100 units/mL recombinant human interleukin-2 (IL-2) for 3 days. Lysis assays were done essentially as described [5]. Briefly, 5×10^4 cells were infected with HSV-1 at an MOI of 10 pfu/cell or were left uninfected. In some cases, 0.1% human gamma globulin (HGG, Gamastan; Miles Canada, Etobicoke) was present throughout the infection and lysis assay to neutralize extracellular virus. For direct lysis assays, fibroblasts were infected for 12–16 h and labeled with ^{51}Cr, and LAK cells were added for 4 h. For indirect (sandwich) lysis assays, cells were infected for 12–16 h, and LAK cells were added for 3 h, after which ^{51}Cr-labeled target K562 cells were added for 6 h. Maximum ^{51}Cr release from target cells was determined by lysis of cells with 1 *M* HCl; spontaneous release of ^{51}Cr was, in every case, <15%. Assays were done in triplicate, and specific lysis was calculated as described previously [17].

Immunofluorescence. A rabbit anti-HSV antiserum (Dako patts, Copenhagen) diluted 1:100 in PBS with 2% bovine serum albumin (PBS-BSA) was preadsorbed against uninfected fibroblasts (pretreated with 2% preimmune goat serum) for 1 h, on ice, three times.

Fibroblasts growing on glass coverslips were infected with 10 pfu/cell HSV-1 (F) or FgDβ or were left uninfected. After 8, 12, or 15½ h of infection, LAK cells were added for 8, 4, or ½ h, respectively. The cells were incubated with 2% preimmune goat serum for 1 h, then with preadsorbed anti-HSV serum for an additional h, all on ice. The cells were washed with cold PBS-BSA and then incubated with fluorescein-conjugated goat anti-rabbit antiserum (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:60 in PBS-BSA, for 45 min on ice. After being washed with PBS-BSA, the cells were fixed using 4% paraformaldehyde for 15 min at room temperature, washed with PBS-BSA, and mounted with 80% glycerol–2.5% 1,4-diazobicyclo[2.2.2]octane [18]. The slides were examined using a confocal microscope (Zeiss, Oberkochen, Germany).

Results

LAK lytic function after contact with cells infected with HSV-1 mutants or exogenous virus. Cells infected with HSV-1 inhibited LAK cell lysis either in direct (not shown) or in sandwich lysis assays, in which LAK were plated with HSV-1–infected human normal fibroblasts or infected Vero cells and subsequently with uninfected ^{51}Cr-labeled K562 target cells (figure 1). These results were similar to those reported by Confer et al. [5]. The pattern of inhibition of lysis in sandwich assays was similar with HSV-infected normal human fibroblasts (figure 1A) and infected Vero cells (figure 1B), except that Vero cells competed with K562 cells for recognition by LAK cells to a much greater extent than did fibroblasts, accounting for the relatively low lysis observed with Vero cells. However, since the HSV-1 mutants used in these experiments have not, for the most part, been characterized in normal fibroblasts, we used Vero cells to test many of the HSV-1 mutants for LAK cell inhibition. Cells

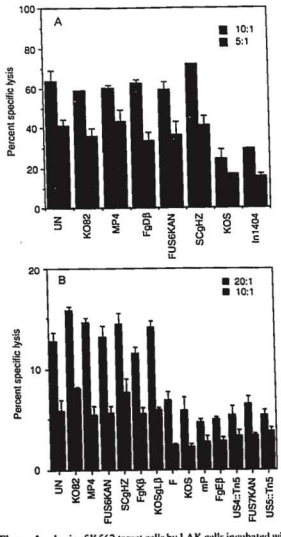

Figure 1. Lysis of K562 target cells by LAK cells incubated with human fibroblasts or Vero cells infected with HSV-1 or with HSV-1 glycoprotein mutants. Normal human fibroblasts (**A**) or Vero cells (**B**) were left uninfected (UN) or were infected with wild-type HSV-1 strains F, KOS, or mP or with HSV-1 mutants KO82 (gB^-), MP4 (gC^-), FUS6KAN (gD^-), FgDβ (gD^-, gI^-), SCgHZ (gH^-), In1404 (gE^-), FgKβ (gE^-), FgEβ (gK^-), KOSgLβ (gL^-), US4::Tn*5* (gG^-), FUS7KAN (gI^-), or US5::Tn*5* (gJ^-) for 12 h. LAK cells were added for 3 h followed by ^{51}Cr-labeled K562 cells for an additional 6 h. Effector-to-target ratios were 20:1, 10:1, or 5:1. Error bars represent 1 SD. Data represent independent experiments **A**, involving fibroblasts, and **B**, involving Vero cells. However, each mutant virus was tested in at least three independent experiments where large fraction of mutant and wild-type viruses were also tested, with similar results.

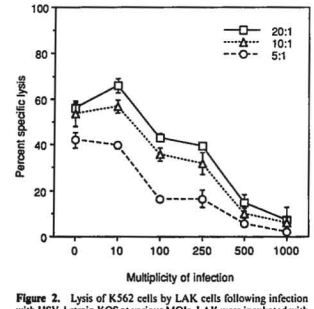

Figure 2. Lysis of K562 cells by LAK cells following infection with HSV-1 strain KOS at various MOIs. LAK were incubated with exogenous HSV-1 for 3 h and then with ^{51}Cr-labeled K562 cells for 6 h. Effector-to-target ratios were 20:1, 10:1, and 5:1. Error bars represent 1 SD.

infected with HSV-1 mutants FgEβ (gE^-), In1404 (gE^-), US4::Tn*5* (gG^-), FUS7KAN (gI^-), or US5::Tn*5* (gJ^-) (figure 1) inhibited lysis by LAK cells to the same extent as did cells infected with wild-type HSV-1.

In contrast, cells infected with the HSV-1 mutants KO82 (gB^-), MP4 (gC^-), FUS6KAN (gD^-), FgDβ (gD^- and gI^-), SCgHZ (gH^-), FgKβ (gK^-), or KOSgLβ (gL^-) (figure 1) did not inhibit lysis compared with uninfected cells. In six different experiments, there were statistically significant differences between the two groups of viruses: group I, including wild-type HSV-1, gE^-, gI^-, gG^- viruses, which can infect cells normally, and group II, including gB^-, gC^-, gD^-, gH^-, gK, and gL^- viruses, which have defects in viral entry. When VD60 cells, which express gD, were infected with the gD^- mutant FgDβ, so that the mutant was complemented, LAK cells were disarmed (results not shown). Effector cells from several donors gave similar results to those shown in figure 1 (not shown).

Confer et al. [5] found that LAK cells infected with exogenous (cell-free) HSV-1 continued to have lytic functions. Similarly, in our experiments LAK cells infected with 10 pfu/cell exogenous HSV-1 for 3 h were unaffected in their ability to lyse K562 cells. However, infection of LAK cells using higher MOIs resulted in progressive inhibition of LAK function (figure 2).

Expression of HSV antigens by LAK cells after contact with HSV-1–infected fibroblasts. Although LAK cells were inactivated after infection with HSV-1 (figure 2), we were uncertain as to the physiologic relevance of this result because high MOIs of exogenous virus were required. HSV-1 normally remains primarily cell-associated and, thus, the high MOIs of exogenous virus used in experiments shown in figure 2 were derived by sonicating infected permissive Vero cells. Immunofluorescent staining was used to test the possibility that direct contact with HSV-1–infected cells resulted in entry of the virus into LAK cells and expression of viral polypeptides. LAK cells that had been incubated for 8 h on fibroblasts infected with wild-type HSV-1 strain F consistently displayed intense surface fluorescence, suggesting a high level of expression of viral polypeptides (figure 3A–D). The fluorescence was HSV-specific because uninfected fibroblasts did not fluoresce. The plane of focus in figure 3B, D, F, and H was adjusted to demonstrate the fluorescence of the lymphocytes, which are spherical and thus appear more fluorescent than the flatter fibroblasts in these confocal micrographs. LAK cells incubated for 4 h on HSV-1 strain F–infected fibroblasts also showed specific immunofluorescence, although at lower levels (figure 3E, F), and we were unable to detect immunofluorescence associated with LAK cells incubated with infected cells for only ½ h (not shown). Lymphocytes adhering to glass coverslips rather than to infected fibroblasts frequently showed no fluorescence (figure 3C, D), further suggesting that infection of LAK cells required direct cell-to-cell contact. In addition, LAK cells adhering to fibroblasts infected with the HSV-1 mutant FgDβ did not express HSV antigens (figure 3G, H), even after 8 h of contact. FgDβ does not express gD and is therefore unable to spread from cell to cell, although this mutant virus was produced on complementing VD60 cells, which supply gD and can thus enter the fibroblasts [13]. As expected, normal human fibroblasts infected with either FgDβ or wild-type HSV-1 strain F showed intense fluorescence (figure 3).

Neutralization of extracellular virus by HGG. To further examine the hypothesis that direct cell-to-cell transfer of HSV-1 into LAK cells accounted for the inhibition of LAK cell function, indirect assays were done in the presence of pooled HGG. HGG can neutralize extracellular virus but does not prevent cell-to-cell spread of HSV-1 and is used in plaque assays to limit secondary plaque production. Gamma globulin had no effect on the inhibition of target cell lysis by LAK in contact with HSV-infected Vero cells (figure 4).

Discussion

In keeping with previous observations [5], we found that cells infected with wild-type HSV induced a dramatic reduction in LAK cell lysis, both of the infected cells themselves and, in indirect (sandwich) lysis assays, of normally highly sensitive K562 cells. This effect was observed only relatively

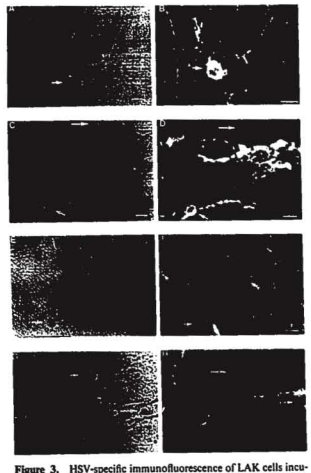

Figure 3. HSV-specific immunofluorescence of LAK cells incubated with fibroblasts. Normal human fibroblasts were infected with wild-type HSV-1 strain F for 8 h (**A–D**) or 12 h (**E, F**) or with HSV-1 gD^- mutant FgDβ for 8 h (**G, H**). LAK cells were incubated with infected fibroblasts for an additional 8 h (**A–D, G, H**) or for 4 h (**E, F**). Cells were placed on ice, stained with rabbit anti-HSV serum and fluorescein-conjugated anti-rabbit antibodies, and fixed. **A, C, E,** and **G**, phase-contrast micrographs of **B, D, F,** and **H. B** and **D**, infected lymphocytes overlying infected fibroblasts (short arrows) displayed intense fluorescence, whereas lymphocyte not in contact with infected fibroblast (**D**, long arrow) did not. **E** and **F**, lymphocyte in contact with infected fibroblast for 4 h displayed weaker, stippled fluorescence (arrow). **G** and **H**, lymphocytes in contact with FgDβ-infected fibroblast did not display HSV-1 antigens (arrows). Bars = 10 μm.

late (i.e., 8–12 h) after HSV-1 infection of the cells (not shown). Cells infected with HSV-1 mutants lacking any of the glycoproteins (gB, gD, gH, gK, and gL) known to be essential for entry of HSV-1 into cultured cells were incapable of inhibiting LAK function, either in direct (not shown) or sandwich lysis assays. Cells infected with HSV-1 mutant viruses lacking so-called "nonessential" glycoproteins (gE, gG, gI, and gJ), that is, those not required for entry into cells, inhibited lysis by LAK cells. Cells infected with a virus lacking gC, considered a nonessential glycoprotein, were also incapable of inhibiting lysis by LAK cells; however, gC enhances virus entry and gC^- viruses replicate much less efficiently than wild-type virus [19]. We note that the MP4 (gC^-) virus produces syncytial plaques on Vero cells but this property did not promote its entry into LAK cells or confer the ability to disarm the cells. These results support the hypothesis that HSV must enter LAK cells to inhibit LAK cell function.

Inhibition of LAK by preparations of exogenous (cell-free) HSV-1 required high concentrations of virus (MOI >100). Thus, LAK cells were inhibited much more effectively after direct contact with HSV-infected cells than by infection with exogenous virus. In these assays, fibroblasts or Vero cells were infected for at least 8 h prior to addition of the LAK cells, which were then left in contact with the infected cells for an additional 2–4 h. HSV-1 infection of fibroblasts for shorter periods (4 h) produced no inhibition of LAK cells (results not shown). During the 10–12 h during which fibroblasts or Vero cells were infected, infectious HSV-1 was presumably produced and entry-competent viruses apparently entered LAK cells. Immunofluorescence experiments verified that viral antigens were produced in LAK cells after contact with infected fibroblasts for 4 h but not in LAK cells in contact with glass coverslips.

Close apposition between membranes of LAK or NK cells and HSV-infected target cells apparently allows efficient entry of HSV into LAK and NK cells. Supporting this hypothesis, neutralizing antibodies were unable to prevent the

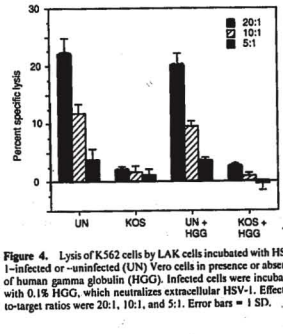

Figure 4. Lysis of K562 cells by LAK cells incubated with HSV-1–infected or –uninfected (UN) Vero cells in presence or absence of human gamma globulin (HGG). Infected cells were incubated with 0.1% HGG, which neutralizes extracellular HSV-1. Effector-to-target ratios were 20:1, 10:1, and 5:1. Error bars = 1 SD.

inhibition of LAK cells. Thus, extracellular virus appears not to play an important role in LAK cell inhibition, confirming the observations of Confer et al. [5] that medium from infected cells was insufficient to inhibit LAK or NK lysis. Further, it appears that the transfer of HSV into LAK cells allows HSV to escape contact with neutralizing antibodies and, thus, the cell-to-cell spread of HSV must occur at tight junctions between the target and effector cells. This is a similar situation to that occurring when HSV spreads between neighboring adherent cells during plaque production (e.g., fibroblast cell monolayers incubated with anti-HSV antibodies). With cultured cells, most HSV remains associated with the infected cell and, frequently, large quantities of virus accumulate on or near the cell surface [20–22]. Therefore, it appears that HSV has developed mechanisms by which viruses can be directly transferred from cell to cell, and these mechanisms of viral dissemination extend to NK and LAK cells, which come into close contact with HSV-infected fibroblasts during the course of immune surveillance. Recurrent HSV infections in humans frequently occur in the presence of high titers of neutralizing antibody (reviewed in [23]), and so direct cell-to-cell spread of HSV in tissues and to the cells of the immune system may be important in vivo.

Activated lymphocytes are known to be more easily infected with HSV than are resting lymphocytes [24, 25]; thus, NK or LAK cells that have recognized a target cell, in this case an HSV-infected cell, may be good hosts for HSV infection. It is not clear whether specific viral gene products act to inhibit LAK function or whether the LAK cells are affected by the initiation of HSV-1 infection and concomitant effects on cellular transcription and translation pathways. A mutant HSV-1 lacking the viral host shutoff protein, vhs [26], inhibited lysis as efficiently as wild-type virus (not shown); therefore, the effect is apparently not because of inhibition of lymphocyte protein synthesis by this gene product.

It is not clear what role the inhibition of LAK or NK lytic function by HSV plays in vivo. If a significant proportion of the local effector population is inhibited in this way, HSV may largely avoid this form of immune surveillance. Similar effects have recently been reported with $CD8^+$ cytotoxic T lymphocytes, which showed profound reductions in function following contact with HSV-infected cells [27, 28], and monocyte function was inhibited after infection with HSV [29]. Cell-to-cell spread of HSV from fully permissive cells to components of the immune system may be a general strategy of HSV pathogenesis and may contribute to the mild immune deficiency that often occurs after HSV reactivation [2–4].

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CHAPTER 1 DISCUSSION

This chapter described the mechanism of inhibition of LAK cells by HSV-infected target cells. During and after this work, it has become clear that inhibition of lytic lymphocytes by infection via cell-to-cell spread is relevant to CD8+ CTL as well as to NK and LAK cells (Posavad and Rosenthal, 1992; Posavad et al., 1993). Moreover, HSV infection of monocytes alters several aspects of monocyte function (Hayward et al., 1993). This suggests that this phenomenon may be of general importance in HSV pathogenesis.

Mouse models are unlikely to be useful in determining the biologic relevance of lymphocyte infection, as mouse LAK and NK cells are not efficiently inactivated in this way. Evidence from human studies is at best circumstantial. Efficient infection of lymphocytes or of monocytes could be one cause of the moderate immunosuppression often seen during HSV reactivation (Sheridan et al., 1982; Rola-Pleszczynski and Lieu, 1984; Vestey et al., 1989). If infection of lymphocytes and/or monocytes occurs in natural infection, then immunohistochemistry should show HSV antigen in these cell types in HSV lesions. No such infection was detected (Cunningham et al., 1985), although the detection technique in this study was not very sensitive. On the other hand, HSV can be detected in blood, not only in immune-compromised patients (Stanberry et al., 1993) but (using PCR) in normal individuals (Cantin et al., 1993), although overt viremia is rarely if ever found during uncomplicated primary herpes infections (Halperin et al., 1983). Thus, while it is possible that HSV does infect infiltrating lymphocytes, some of which then escape into the peripheral circulation, clear evidence is lacking. It is important to note that the only known case of a human lacking NK cells came to light because the individual suffered

recurrent herpesvirus (including HSV) infections (Biron et al., 1989), which argues that NK cells normally do play a role in limiting HSV infection and reactivation.

Another as yet unanswered question is the mechanism by which HSV infection of lymphocytes results in inactivation of lytic function. HSV-infected lymphocytes are not rapidly killed, and most cells are abortively infected (Braun et al., 1984; Sarmiento and Kleinerman, 1990), so the inhibition cannot be explained simply by effector cell death. However, even abortive infection of cells with HSV can lead to cytopathic effects (Johnson et al., 1992), including effects which could readily inhibit lysis: cytoskeletal alterations (Heeg et al., 1986), loss of adhesion molecules (Dienes et al., 1985) and changes in transcription and translation. It is also possible that the inhibition is blocked specifically. If so, the gene product(s) are most likely to be immediate-early genes or structural proteins, because LAK cells and CTL are inactivated quite rapidly following exposure to infected targets. In fact, experiments suggest that IE gene products or structural proteins are involved (Posavad et al., 1994).

In theory, experiments aimed at further identifying the gene product(s) involved in inhibition of lytic cell function are fairly straightforward. Infection of lytic cells with mutant viruses lacking various IE proteins, and with virus inactivated by UV crosslinking, could be used to identify essential gene products. However, not only are some of the IE mutant viruses technically difficult to work with, the function may involve multiple gene products, as with the induction of cytopathic effects (Johnson et al., 1992). Mutant HSV lacking more than one IE gene are not available; and while some double or multiple mutant viruses could be constructed, several double-IE mutant viruses are likely to be severely impaired

for growth, even on cell lines providing one of the IE proteins in *trans*. Thus, further identification of the gene(s) involved may be difficult.

The general purpose of this thesis was to identify mechanisms by which HSV avoids clearance by the host immune system. Resistance to lysis by LAK, NK, and CTL by inhibiting function due to cell-to-cell spread of the virus is one such mechanism. However, as noted above, this effect can only occur late in the infectious cycle of HSV. Since preventing lysis early in infection would be more advantageous for the virus, I also investigated mechanisms of immune evasion during the early stages of infection. The results of these investigations are discussed in the next chapter.

CHAPTER 2

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A Cytosolic Herpes Simplex Virus Protein Inhibits Antigen Presentation to CD8+ T Lymphocytes

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Summary

Herpes simplex virus (HSV) infection of human fibroblasts rapidly renders the cells resistant to lysis by HSV-specific CD8+ cytotoxic T lymphocytes (CTLs), which normally recognize cell surface major histocompatibility complex (MHC) class I proteins presenting viral peptides. Within 3 hr of infection with HSV, MHC class I protein complexes are retained in the endoplasmic reticulum (ER)/cis Golgi and show properties of complexes lacking antigenic peptide. The HSV immediate-early protein ICP47 is both necessary and sufficient to block transport of class I proteins and to inhibit lysis by CD8+ CTLs. The target for ICP47 is not known, but since ICP47 does not associate with membranes, it appears that ICP47 inhibits the production or stabilization of antigenic peptides or their translocation into the ER/cis Golgi. Thus, by expressing ICP47, HSV can evade detection by CD8+ T lymphocytes, perhaps explaining the predominance of CD4+ rather than CD8+ HSV-specific CTLs in vivo.

Introduction

T lymphocytes recognize short peptides derived from viral, bacterial, or cellular proteins and presented on cell surface major histocompatibility (MHC) proteins (reviewed by Yewdell and Bennink, 1992). The expression of MHC class II proteins is restricted to specialized cell types, and these molecules present peptides, usually derived by proteolysis of endocytosed proteins, to CD4+ T lymphocytes. MHC class I proteins are expressed on most cell types and present peptide fragments of cytosolic proteins to CD8+ T lymphocytes. Cellular and viral peptides destined to be presented on MHC class I appear to be generated by ubiquitin-dependent proteasomes in the cytoplasm (Michalek et al., 1993). These peptides are then transported by ATP-dependent TAP transporter proteins from the cytosol into the endoplasmic reticulum (ER) or cis-Golgi apparatus (Shepherd et al., 1993; Androlewicz et al., 1993), where the peptides bind to nascent MHC class I heavy chains that noncovalently associate with β2-microglobulin (β_2m). The resulting trimolecular complex is transported to the cell surface and recognized by T lymphocytes (reviewed by Yewdell and Bennink, 1992). In mutant cell lines lacking the TAP transporter proteins, MHC class I proteins lack peptides and are consequently misfolded, unstable, and retained in the ER/cis Golgi (Townsend et al., 1989; Ljunggren et al., 1990; Baas et al., 1992).

A number of animal viruses have been found to evade T lymphocyte recognition by down-regulating MHC class I expression or by expressing proteins that alter or block the MHC class I antigen presentation pathway (Gooding, 1992; McFadden and Kane, 1993). This interference with T cell recognition of infected cells may play a critical role in enabling these viruses to persist or to cause recurrent infections. For example, adenovirus type 2 (Ad2) and type 5 (Ad5) express a 19 kDa glycoprotein that binds to MHC class I proteins causing retention in the ER/cis Golgi (Burgert and Kvist, 1985; Andersson et al., 1985). Murine cytomegalovirus (MCMV) and human cytomegalovirus (HCMV) reduce cell surface MHC class I by different mechanisms: MCMV causes peptide-loaded MHC class I molecules to accumulate in the Golgi apparatus (del Val et al., 1992), while HCMV causes MHC class I heavy chains to be unstable and degraded (Beersma et al., 1993). With the exception of adenoviruses, viral polypeptides responsible for these effects have not been identified, which is unfortunate because these proteins could be invaluable tools in the analysis of antigen presentation pathways.

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are common human pathogens that first infect mucosa and then spread via sensory neurons into ganglia, where they establish lifelong latent infections. During periods of latency, HSV is relatively resistant to host immune responses. Reactivation of HSV from neurons and reinfection of mucosal tissues occur intermittently despite the presence of relatively high titers of neutralizing antibody (Zweerink and Stanton, 1981). The relative insensitivity of HSV to antiviral antibodies is partly explained by the ability of the virus to spread directly from cell to cell and by HSV-encoded complement and Fc receptors, which may protect the virus from antibody-mediated cytolysis (Friedman et al., 1984; Johnson et al., 1988; Frank and Friedman, 1989). Moreover, reactivation of HSV is frequently associated with moderate suppression of cellular immune responses (Vestey et al., 1989), suggesting that HSV may also interfere with T lymphocyte recognition.

Several investigators have observed that anti-HSV cytotoxic T lymphocytes (CTLs) in humans are disproportionately represented by the CD4+ T cell phenotype rather than the more common CD8+ phentotype (Schmid and Rouse, 1992). This may be related to the unusual observation that human fibroblasts infected by HSV are not recognized by HSV-specific CD8+ CTLs (Posavad and Rosenthal, 1992; Koelle et al., 1993). Resistance of human fibroblasts to HSV-specific CTL recognition is observed within a few hours after infection, before cell surface MHC class I is reduced by virus-induced inhibition of host protein synthesis. At these early times after infection, there appears to be a defect in presentation of viral antigens to CTLs because exogenous addition of the relevant HSV peptides, which bypasses antigen presentation pathways, renders HSV-infected fibroblasts sensitive to lysis by anti-HSV CD8+ CTLs (Koelle et al., 1993; M. A. Tigges et al., unpublished data). These results also indicate that the HSV-infected fibroblasts are not inherently resistant to CTL lysis.

Here we report that human and murine MHC class I proteins produced in HSV-infected human fibroblasts are unstable and are retained in the ER/cis Golgi. We identified an HSV immediate-early protein, ICP47, that was both necessary and sufficient to cause MHC class I retention in the ER/cis Golgi and to reduce lysis of human fibroblasts by CD8+ CTLs.

Results

Murine CTLs Are Unable to Lyse Human Cells Infected with an HSV-1 Expressing Murine MHC Class I Protein

Although previous results suggested that human CD8+ CTLs do not recognize HSV-infected human fibroblasts, CD8+ CTLs derived from HSV-infected mice readily lyse syngeneic HSV-infected mouse fibroblasts or other mouse cells (Pfizenmaier et al., 1977; I. A. Y., unpublished data). To understand these observations better, we found it of interest to determine whether HSV-infected human fibroblasts expressing murine MHC class I molecules could be lysed by murine HSV-specific CTLs. A recombinant HSV, F-US5MHC, that expresses murine MHC class I (H-2K^b) was constructed by placing the H-2K^b heavy chain and murine β_2m genes under the control of HSV promoters for ICP6 and thymidine kinase, respectively, and by inserting the genes into the *US5* gene. This virus expressed both proteins in human cells, and the recombinant proteins reacted with the conformation-specific monoclonal antibody (MAb) Y3, indicating that a complex of H-2K^b and β_2m was formed (data not shown).

Infection with F-US5MHC should in theory render any cell susceptible to lysis by murine H-2^b-restricted anti-HSV CTLs. Regardless of their haplotype, mouse cells infected with F-US5MHC were efficiently lysed by murine H-2^b-restricted CTLs, while with wild-type HSV-1 or a control virus, F-US5βsyn (in which *lacZ* sequences were inserted into *US5*), only cells of the H-2^b haplotype were lysed (Figures 1A and 1B). In contrast, normal human fibroblasts that had been infected with F-US5MHC were not lysed by HSV-specific H-2^b-restricted CTLs (Figure 1C). These results, coupled with previous observations with peptide-treated fibroblasts, suggest that there is a defect in antigen presentation to T lymphocytes in HSV-infected human fibroblasts that is not related to inhibition of MHC class I synthesis and that affects both human and mouse MHC class I in these cells.

MHC Class I Molecules in HSV-Infected Human Fibroblasts Are Retained in the ER/Cis Golgi

MHC class I protein complexes must be transported to the cell surface to present viral peptides to T lymphocytes.

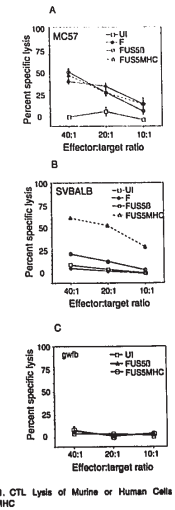

Figure 1. CTL Lysis of Murine or Human Cells Infected with F-US5MHC

HSV-specific CD8+ CTLs were derived from C57BL/6 mice (H-2^b) infected with HSV-1 strain F. Lysis of mouse MC57G cells (H-2^b) (A), mouse Sv/BALB cells (H-2^d) (B), or normal human fibroblasts (gwfb) (C), which were left uninfected (UI) or were infected with wild-type HSV-1 (F), F-US5MHC, or F-US5βsyn. Percent specific lysis of the target cells was determined as described in Experimental Procedures.

To examine cell surface transport of MHC class I complexes in HSV-infected cells, we radiolabeled the cells using a pulse–chase protocol, and then heavy chains were immunoprecipitated and digested with endoglycosidase H (endo H). Conversion of the class I heavy chains from an endo H–sensitive form to an endo H–resistant form occurs in the Golgi apparatus (Townsend et al., 1989). In uninfected cells, MHC class I heavy chains became resistant to endo H during a 90 min chase, while MHC class I from HSV-1- or HSV-2-infected cells remained sensitive to endo H (Figure 2A). MHC class I was selectively affected, since HSV glycoprotein D (gD) and the transferrin receptor were efficiently converted to endo H–resistant forms during the chase period in infected cells (Figures 2B and 2C). Inhibition of class I processing was first observed

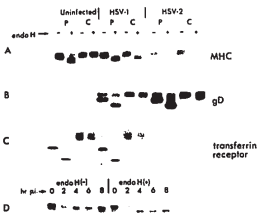

Figure 2. MHC Class I Remains Endo H–Sensitive in HSV-Infected Human Fibroblasts

Human fibroblasts (gwfb) were left uninfected or were infected with HSV-1 strain KOS or HSV-2 strain 333 for 3 hr and then labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min and lysed (P, pulse) or the label chased for 90 min before lysis (C, chase) and subsequently treated with endo H (plus) or by mock treatment (minus).

(A) MHC class I heavy chain was immunoprecipitated from detergent extracts of the cells using MAb HC10.

(B) HSV-1 or HSV-2 gD was immunoprecipitated using MAb LP2.

(C) The transferrin receptor was immunoprecipitated using MAb T56/14.

(D) Human fibroblasts were infected with HSV-1 for 0, 2, 4, 6, or 8 hr and then radiolabeled for 30 min, and, in every case, the label was chased for 90 min. MHC class I was immunoprecipitated with W6/32, and samples were either digested with endo H (minus) or mock digested (minus) before electrophoresis and autoradiography.

within 2 hr after infection with HSV-1, and the effect was complete by 4 hr (Figure 2D). Consistent with the observed differences in susceptibility to CD8+ CTLs, mouse H-2K^b class I molecules expressed in human fibroblasts infected with F-US5MHC also remained in an endo H–sensitive form, while H-2K^b and other mouse MHC class I proteins expressed in HSV-infected mouse cells became endo H resistant (data not shown). The results demonstrate that MHC class I complexes are retained in the ER/cis Golgi of human fibroblasts soon after infection with either HSV-1 or HSV-2.

MHC Class I Complexes in HSV-Infected Cells Are Misfolded and Unstable

MHC class I complexes produced in TAP$^-$ cells do not contain peptide, are retained in the ER/cis Golgi, and are unstable and inefficiently recognized by certain MAbs (Townsend et al., 1989). It appeared possible that MHC class I complexes in HSV-infected cells might lack peptide and therefore be misfolded and unstable. The stability of MHC class I complexes from HSV-infected human fibroblasts was investigated by using two anti–heavy chain MAbs: MA2.1, which recognizes only properly folded heavy chain–light chain complexes of the histocompatibility leukocyte antigen HLA-A2 haplotype, and HC10, which, under the conditions used, recognizes misfolded as well as folded MHC class I heavy chains.

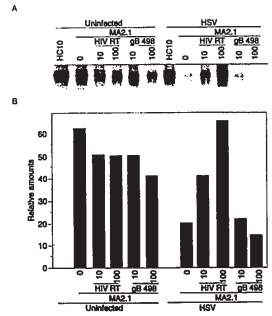

Figure 3. MHC Class I Proteins in HSV-Infected Cells Are Misfolded and Unstable

(A) MRL fibroblasts (HLA-A2 homozygous), left uninfected or infected with HSV-1 VhsB for 4 hr, were radiolabeled for 1 hr. Extracts of the cells were incubated for 18 hr at 4°C with 0, 10, or 100 μM HIV reverse transcriptase peptide 476–484 (HIV RT), which binds to HLA-A2, or with peptide gB498-505, which does not interact with HLA-A2 (gB 498). MHC class I proteins were immunoprecipitated with MAb HC10, which recognizes both free and β_2m-bound heavy chains under the conditions used, or with MA2.1, which recognizes only HLA-A2 heavy chain–β_2m complexes. Bands corresponding to MHC class I heavy chains are shown.

(B) HLA-A2 proteins precipitated by MAb MA2.1 were quantified and plotted relative to the amount of MHC class I precipitated by HC10 (arbitrarily set at 100).

Human fibroblasts were left uninfected or were infected with an HSV-1 mutant, *VhsB*, lacking the virion host shutoff gene product, to minimize differences in MHC class I synthesis between infected and uninfected fibroblasts. Detergent extracts of the cells were incubated for 18 hr in the presence of a peptide encompassing human immunodeficiency virus (HIV) reverse transcriptase residues 476–484, which binds to HLA-A2; with a control peptide, gB498-485, which does not interact with HLA-A2; or in the absence of added peptide and then MHC class I proteins immunoprecipitated with MAb MA2.1 or HC10. There was no reduction in the total MHC class I (precipitated by HC10) produced in HSV-infected cells (Figure 3) and no evidence for proteolysis of class I during the 18 hr incubation (data not shown). However, HLA-A2 produced in HSV-infected fibroblasts was inefficiently recognized by MA2.1. Addition of the HIV reverse transcriptase 476–484 peptide to extracts of HSV-infected cells increased the stability of HLA-A2, compared with lysates with no peptide or control pep-

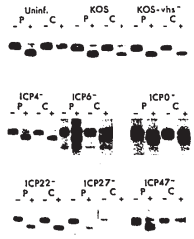

Figure 4. An HSV ICP47 Mutant Does Not Inhibit Cell Surface Transport of MHC Class I Proteins

Human fibroblasts were left uninfected or were infected with wild-type HSV-1 strain KOS or with the following HSV-1 mutants: *KOS-vhs⁻*, which lacks the virion host shutoff gene; *d120* (ICP4⁻); *hr83* (ICP6⁻); *dx2.1* (ICP0⁻); *R325-9TK⁻* (ICP22⁻); *5d1.2* (ICP27⁻); or *N38* (ICP47⁻). After 3 hr, an endo H pulse (P)–chase (C) protocol was performed as described in Figure 2. In some instances, a contaminating cellular protein of ~45 kDa was also precipitated; however, this protein was insensitive to endo H and did not obscure the faster-migrating endo H digestion product of MHC class I.

tide added (Figure 3). A similar degree of instability was found with total cellular MHC class I, measured with MAb W6/32, which reacts with all HLA-A, HLA-B, and HLA-C molecules complexed with β_2m (data not shown). As well, mouse H-2K^b class I proteins were stabilized in cell extracts derived from F-US5MHC-infected human fibroblasts by addition of the gB498-50 peptide, which binds to H-2K^b (data not shown). Therefore, the instability of the MHC class I complexes is largely rectified by the addition of antigenic peptide, supporting the hypothesis that class I complexes in HSV-infected fibroblasts lack peptide.

HSV-1 Immediate-Early Gene Product ICP47 Is Required for ER Retention of MHC Class I

HSV expresses three temporal classes of gene products: immediate-early, early, and late, with synthesis of the early and late proteins being dependent on immediate-early gene products (reviewed by Roizman and Sears, 1990). Also, a number of HSV proteins are structural components of the virus particle and are delivered into host cells upon virus entry. Since MHC class I complexes were retained in the ER within 2 hr following HSV infection (see Figure 2D), it was likely that the block was due to either a virion structural protein or an immediate-early gene product. Inhibition due to an immediate-early viral gene product would require de novo synthesis of viral proteins. To determine whether synthesis of HSV polypeptides was required for the effect, we subjected HSV-2 preparations to ultraviolet inactivation so that cells infected with the viruses did not express viral proteins. Fibroblasts were treated with relatively large quantities (~5000 particles/cell) of ultraviolet-inactivated HSV-2, and no effect on MHC class I protein transport was observed (data not shown). Therefore, HSV structural proteins are not sufficient to inhibit class I transport.

To evaluate potential roles of HSV immediate-early proteins in this effect, we analyzed a panel of HSV-1 mutants, each unable to express one of the six immediate-early proteins. In cells infected with HSV-1 *d120*, a mutant unable to express the immediate-early protein ICP4, MHC class I remained endo H sensitive (Figure 4). Since ICP4 is strictly required for expression of both early and late proteins (Dixon and Schaffer, 1980; Watson and Clements, 1980), *d120* expresses only immediate-early proteins, implying that expression of an immediate-early protein other than ICP4 is sufficient to block MHC class I transport. Mutants lacking immediate-early proteins ICP0, ICP6, ICP22, or ICP27 were all able to block processing of MHC class I (Figure 4), indicating that these immediate-early proteins were not required. In contrast, MHC class I proteins were processed and therefore were not retained in the ER/cis Golgi in cells infected with mutant *N38*, which is unable to express HSV immediate-early protein ICP47. Besides its defect in the *ICP47* gene, *N38* also lacks coding sequences for the *US9*, *US10*, and *US11* genes. However, the *US9*, *US10*, and *US11* genes are early and late genes and would not be expressed in cells infected with the ICP4⁻ virus *d120*, in which processing of MHC class I was blocked, and therefore these genes cannot be required for this effect. Moreover, a second mutant, *R3631*, lacking only the *ICP47* and *US11* genes, also failed to block MHC class I processing (data not shown). *ICP47* is an immediate-early gene that does not require ICP4 for its expression and has no known effect on virus replication or transcription (Umene, 1986; Mavromara-Nazos et al., 1986), and thus expression of the other immediate-early proteins, as well as early and late proteins, in cells infected with *ICP47* mutants is normal. Therefore, ICP47 is essential for HSV-induced inhibition of MHC class I transport to the cell surface, and expression of other HSV genes is not required.

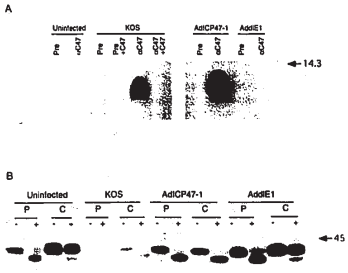

Figure 5. ICP47 Expressed Using a Recombinant Adenovirus Vector Alters MHC Class I Processing

(A) Human fibroblasts were left uninfected or infected with HSV-1 strain KOS using 10 pfu/cell or AdICP47-1 or AddIE1 using 100 pfu/cell. The cells were radiolabeled for 2 hr (2–4 hr postinfection with HSV or 34–36 hr with adenovirus vectors). Cell extracts were mixed with an ICP47-specific serum (αC47) or with preimmune serum (Pre) or with anti-C47 antibodies preincubated with the C47 peptide (αC47 plus C47). A 14.3 kDa marker protein is indicated. (B) Fibroblasts were left uninfected or were infected with HSV-1 strain KOS, AdICP47-1, or AddIE1, and after 4 hr infection with HSV-1 or 36 hr infection with the adenovirus vectors, an endo H pulse (P)–chase (C) protocol was performed as described in Figure 2. A 45 kDa marker is indicated.

Expression of HSV-1 ICP47 Using an Adenovirus Vector Prevents MHC Class I Transport and Processing

To confirm and characterize further the effects of ICP47, we constructed a recombinant adenovirus, AdICP47-1, in which HSV-1 *ICP47* coding sequences were coupled to the HCMV immediate-early promoter and inserted into the E1 region of Ad5. Since E1⁻ adenoviruses are replication defective, AdICP47-1 does not express significant quantities of Ad5 proteins or cause cytopathic effect in human cells. Fibroblasts infected with AdICP47-1 expressed high levels of ICP47 when compared with HSV-infected cells (Figure 5A). MHC class I produced in human fibroblasts infected with AdICP47-1 remained sensitive to endo H, while MHC class I from uninfected cells became largely endo H resistant (Figure 5B). MHC class I was also transported through the Golgi in cells infected with AddIE1, which contains identical deletions in the E1 and E3 regions of Ad5 as are present in AdICP47-1.

Both AdICP47-1 and AddIE1 contain a small deletion or substitution in the E3 region of Ad5, derived from Addl309, that leaves the coding sequences for the 19 kDa MHC class I–binding glycoprotein intact (Jones and Shenk, 1979). However, because AdICP47-1 and AddIE1 lack E1 sequences, E3 proteins are not expressed in human cells (Jones and Shenk, 1979), and both viruses have identical deletions in the E1 and E3 regions. In addition, MHC class I transport was specifically inhibited in fibroblasts infected with a second adenovirus vector, AdICP47-3, in which ICP47 was inserted into the E3 region so that the coding sequences for the 19 kDa glycoprotein gene are deleted (data not shown). Therefore, ICP47, in the absence of other HSV polypeptides, can inhibit cell surface transport of class I. The MHC class I complexes produced in AdICP47-1-infected fibroblasts were also misfolded and unstable (data not shown).

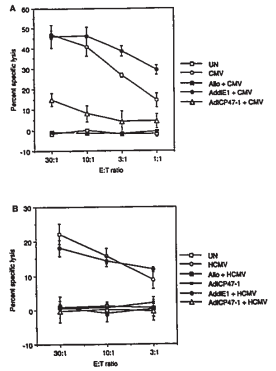

Figure 6. Human Fibroblasts Expressing ICP47-1 Are Inefficiently Lysed by CD8⁺ CTL Clones

Human fibroblasts fibroblasts were left uninfected (UN) or infected with AdICP47-1 (150 pfu/cell) or AddIE1 (150 pfu/cell) for 24 hr and then infected with HCMV (5 pfu/cell) for 12 hr. Other fibroblasts were infected with HCMV without prior infection with adenovirus vectors (HCMV) or infected with AdICP47-1 alone (AdICP47-1). In (A) MR fibroblasts were mixed with CTL clone MR-16E8 (HLA-B35 restricted), which is specific for the HCMV protein pp65. In (B) DG fibroblasts were mixed with CTL clone DG-28D3, which is specific for an unknown HCMV protein. Allo plus HCMV indicates allogeneic DG fibroblasts infected with HCMV in (A) and MR fibroblasts infected with HCMV in (B). Percent specific lysis of the target cells was calculated as described in Experimental Procedures. E:T, effector:target.

Expression of HSV-1 ICP47 Inhibits Lysis of Human Cells by CD8⁺ CTLs

If human fibroblasts expressing ICP47 are unable to transport MHC class I to the cell surface, then the cells should be unable to present viral peptides to T lymphocytes and be resistant to anti-HSV CTLs. To test this, we attempted to determine whether human anti-HSV CD8⁺ CTLs would more efficiently recognize fibroblasts infected with HSV ICP47 mutants. However, the results of these experiments were inconclusive, primarily owing to the fact that human CD8⁺ CTLs, obtained from seropositive donors, have only low levels of anti-HSV cytolytic activity.

As an alternative approach, we tested the ability of ICP47, expressed using AdICP47-1, to alter lysis of HCMV-infected fibroblasts by HCMV-specific CD8⁺ CTL clones. In the experiments shown (Figures 6A and 6B), human fibroblasts were infected with AdICP47-1 or AddIE1 for 24 hr and then superinfected with HCMV for 12 hr. Target cells were then tested in ^{51}Cr release assays with either clone MR-16E6, which is specific for HCMV protein pp65 and is restricted by HLA-B35 (Figure 6A), or clone DG-28D3, which is specific for an unknown HCMV antigen and restricted by HLA-B14 (Figure 6B) (Riddell et al., 1991). With both clones, lysis of fibroblasts that were infected with AddIE1 and then superinfected with HCMV was similar to that observed with targets infected with HCMV alone. In contrast, preinfection of fibroblasts with AdICP47-1 markedly reduced lysis of HCMV-infected cells by both CTL clones (Figures 6A and 6B). Lysis of HCMV-infected allogeneic cells and of AdICP47-1-infected fibroblasts did not differ from uninfected cells. Competition between peptides derived from ICP47 and the HCMV proteins for MHC class I presentation is unlikely because ICP47 inhibited both CTL clones, which are restricted by different MHC class I molecules. Moreover, the amino acid sequence of ICP47 does not contain peptides that match the motif for HLA-B35 (Falk et al., 1993). The MR-16E6 clone also lyses fibroblasts infected with Vacpp65, a vaccinia virus vector expressing HCMV pp65, and prior infection with AdICP47-1 markedly inhibited lysis of Vacpp65-infected fibroblasts (data not shown). These observations demonstrate that ICP47 not only causes MHC class I molecules to be retained in the ER in an unstable form, but also markedly inhibits recognition by CD8⁺ T lymphocytes.

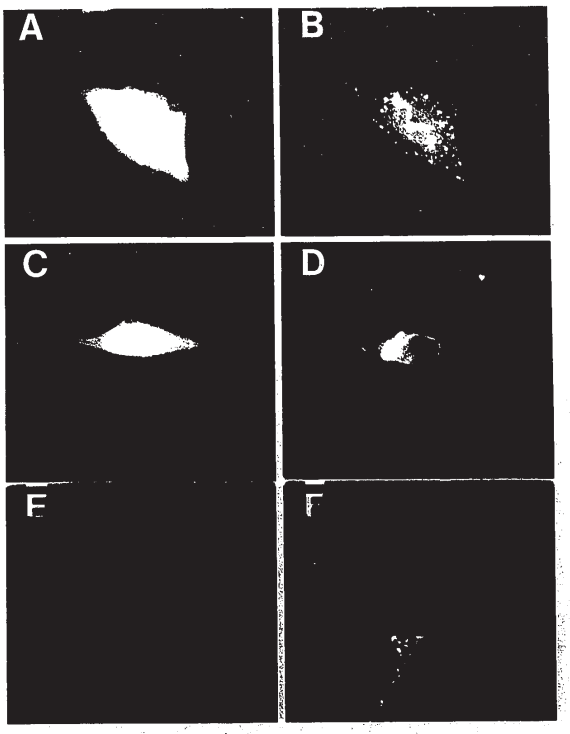

Figure 7. Immunofluorescent Staining of ICP47 and MHC Class I Proteins

Human fibroblasts were infected with AdICP47-1 for 24 hr (A and B) or for 36 hr (C and D) or were left uninfected (E and F) and then fixed and permeabilized. The cells were incubated with a mixture of anti-ICP47 rabbit serum and anti–MHC class I MAb and then washed and incubated with a mixture of fluorescein-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG antibodies. The cells were washed and then fluorescein fluorescence associated with ICP47 (A, C, and E) or rhodamine fluorescence associated with HLA (B, D, and F) photographed using the appropriate filters.

Discussion

Previous studies have shown that HSV-infected human fibroblasts are not lysed by HSV-specific CTLs, although these CTLs could lyse HSV-infected fibroblasts treated with appropriate peptide antigens (Koelle et al., 1993; M. A. Tigges et al., unpublished data). These results suggested that HSV causes defects in antigen presentation to CD8⁺ T lymphocytes shortly after infection. We found that HSV infection of human fibroblasts led to a rapid inhi-

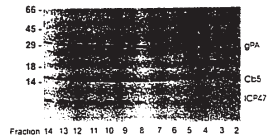

Figure 8. Membrane Binding of ICP47 Translated in Vitro

ICP47; Cb5, which is capable of associating with ER membranes; and gPA, a cytosolic protein, were synthesized in a rabbit reticulocyte cell free system. The proteins were mixed with human KB ER membranes for 15 min at 24°C and then the mixture was subjected to gel filtration using Sepharose CL2B. Fractions collected are indicated along the bottom of the figure: excluded fractions containing membranes were fractions 3–5, while included fractions containing cytosolic proteins were 7–14. The lower part of the figure, containing ICP47 samples, was exposed seven times longer than the upper part of the figure.

riched for either ER or mitochondria (as a control for nonspecific binding) were examined by electron microscopy and by Western blot analysis that indicated that the ER fraction contained a substantial fraction of total cellular MHC class I (data not shown). In vitro translation reactions were incubated with membranes and then subjected to gel filtration to separate soluble, cytosolic proteins (included fraction) from membrane-associated proteins (excluded fraction). ICP47 was found exclusively in cytosolic fractions (7–14), as was a control protein, gPA, which does not interact with membranes (Janiak et al., 1994b) (Figure 8). In contrast, a fraction of the cytochrome b5 (Cb5), which is known to bind to ER membranes (Janiak et al., 1994a), was present in the excluded fractions and was therefore membrane associated. In the experiment shown, only a fraction of the total ICP47 was added to membranes to ensure that membranes were in excess. Densitometric analysis showed that there were more Cb5 molecules associated with the ER membranes than the total number of ICP47 molecules added to the reaction. Thus, it is unlikely that even a small fraction of ICP47 can interact with these membranes. Similarly, ICP47 did not interact with other membrane fractions enriched for mitochondria or dog pancreatic membranes (data not shown). These results indicate that ICP47 does not significantly bind to human ER or to other membranes and is uniformly distributed in the cytoplasm and nucleus of cells.

HSV-1 ICP47 Inhibits Lysis of Human Cells by CD8⁺ CTLs

We initially attempted to assess the capacity of ICP47 to associate with cellular membranes by cell fractionation. The results of these experiments were inconclusive because ICP47 was rapidly degraded in cell homogenates. Instead, ICP47 was synthesized in vitro using a reticulocyte lysate system and was added directly to membrane fractions prepared from human KB cells. Fractions en-

bition of intracellular antigen processing pathways, causing prolonged and specific retention of newly synthesized MHC class I proteins in the ER/cis Golgi. HSV mutants unable to express the immediate-early protein ICP47 failed to alter MHC class I transport, and adenovirus vectors expressing ICP47 caused MHC class I to be retained in the ER, indicating that ICP47 was both necessary and sufficient for this inhibition. Finally, expression of ICP47 in HCMV-infected fibroblasts resulted in a dramatic reduction in sensitivity to HCMV-specific CTLs.

One possible mechanism for retention of MHC class I in the ER involves binding of ICP47 to MHC class I complexes, by analogy to the adenovirus 19 kDa glycoprotein that prevents egress from the ER (Burgert and Kvist, 1985; Andersson et al., 1985). However, a direct interaction between the ICP47 and MHC class I proteins appears unlikely for two reasons. First, both murine and human MHC class I proteins were retained in the ER/cis Golgi in HSV-infected human fibroblasts, yet in HSV-infected mouse fibroblasts, the endogenous MHC class I molecules were transported to the cell surface and presented viral antigens to murine HSV-specific CTLs. If inhibition of antigen presentation was caused by binding to MHC class I, the same MHC class I molecules should be affected, regardless of the cell type. Second, ICP47 lacks an obvious signal sequence (Murchie and McGeoch, 1982), and we found that ICP47 was localized uniformly in the cytoplasm and nucleus, whereas MHC class I proteins were distributed in a more punctate fashion and associated with perinuclear and surface membranes. Moreover, ICP47 translated in vitro did not associate significantly with human KB ER membranes, though the membranes were in vast excess and contained MHC class I proteins. Therefore, it is unlikely that ICP47 reaches the lumen of the ER or even that the protein interacts with the cytoplasmic domain of class I. It remains possible that ICP47 binds to membranes through adaptor proteins that are not components of these membranes.

The most probable explanation for the HSV-mediated inhibition of antigen processing is that ICP47 blocks either the production of peptides in the cytoplasm or their translocation into the ER. As well as being retained in the ER, newly synthesized MHC class I complexes were unstable in HSV-infected cells and were stabilized by addition of the appropriate antigenic peptides. These properties are reminiscent of T2 and RMA-S cells, which do not assemble peptide with MHC class I because they lack the TAP peptide transporter proteins (Townsend et al., 1989; Ljungren et al., 1990; Baas et al., 1992) and support the view that, in HSV-infected cells, assembly of peptide epitopes with the MHC class I heavy chain and β_2m is inhibited. However, since ICP47 is not membrane associated, it is unlikely that the protein alters peptide transfer onto MHC class I from within the ER or that ICP47 interacts directly with TAP transporter proteins. Instead, it is more likely that ICP47 affects the process by which proteins are degraded into peptide epitopes and transported across the cytoplasm to TAP transporter proteins. A most interesting notion is that ICP47 interacts with as-yet-unknown components of the antigen processing pathway, for example, proteins that can stabilize or convey peptides in the cytoplasm. Studies are in progress to characterize the mode of action of ICP47 further, with the expectation that ICP47 will prove very useful in investigating antigen processing pathways in a variety of cell types and in vivo.

There have been recent reports that two other herpesviruses, HCMV and MCMV, block antigen presentation. However, it appears that these viruses produce effects different from those described here: MCMV infection causes MHC class I–peptide complexes to be retained in the medial Golgi (del Val et al., 1992), and HCMV infection causes MHC class I heavy chains to be destabilized and rapidly degraded in the ER (Beersma et al., 1993). Therefore, at the present time, it appears that HSV, HCMV, and MCMV employ different strategies to inhibit antigen presentation pathways. However, until the HCMV and MCMV proteins responsible for these effects are identified, it will be difficult to understand fully and to compare the inhibition of antigen presentation by the different viruses. We note that searches of protein data bases have not yielded obvious homologs of HSV ICP47 in other viruses or among cellular sequences.

The effects of ICP47 in human fibroblasts appear to be most consequential during early phases of HSV infection because HSV shuts off synthesis of host proteins, including MHC class I proteins, by late in the infection (Smibert and Smiley, 1990). In addition, infectious HSV produced in fibroblasts can inactivate CTL, NK, and LAK cells by cell-to-cell spread, and, again, this effect is only observed late after infection (York and Johnson, 1993; Posavad et al., 1993). The combined effects of these early and late inhibitory mechanisms probably contribute substantially to the ability of HSV to survive in vivo, especially during recurrent infections.

In contrast with infected fibroblasts, HSV-infected human B cell lines can be lysed by HSV-specific $CD8^+$ CTLs (Posavad and Rosenthal, 1992; Koelle et al., 1993), indicating that ICP47 is unable to block antigen presentation in these cells. The reasons for this difference are not clear. B cells are not normally hosts for HSV, and infection in these cells is inefficient (Rinaldo et al., 1978), whereas human fibroblasts are productively and efficiently infected by HSV. Thus, in inappropriate host cells, ICP47 may be expressed at inadequate levels or may reach adequate levels too late to prevent presentation of newly synthesized viral antigens. Alternatively, antigen presentation pathways may be quantitatively or qualitatively different in B lymphocytes. However, it appears that ICP47 can inhibit MHC class I presentation in human adherent cell types other than fibroblasts because, for example, a human epithelial carcinoma cell line infected with F-US5MHC was not lysed by murine HSV-specific $CD8^+$ CTLs. Similarly, one could argue that insufficient quantities of ICP47 are expressed in HSV-infected mouse cells; however, we found that ICP47 expression is similar in HSV-infected mouse and human cells (data not shown). Thus, it appears that cellular targets for ICP47 differ between mouse and human cells. Interestingly, mouse cells infected with HSV-2 are less susceptible to lysis by HSV-specific $CD8^+$ CTLs than are cells infected with HSV-1, and this difference maps to the U_s region of the HSV-2 genome, which includes the *ICP47* gene (Carter et al., 1984; McGeoch et al., 1985). HSV-2 ICP47 may be more effective than HSV-1 ICP47 in inhibiting mouse antigen presentation pathways.

Our observations may explain some unusual features of the cellular immune response to HSV: the apparent importance of NK cells for the control of HSV infections (Biron et al., 1989) and the relative paucity of $CD8^+$ CTLs compared with $CD4^+$ CTLs (reviewed by Schmid and Rouse, 1992). This apparent skewing of the normal $CD8^+/CD4^+$ ratio in the CTL population is, perhaps, the best evidence that ICP47 can have important effects in vivo. Those human $CD8^+$ CTL clones that can be established from human HSV lesions predominately recognize viral structural proteins rather than the products of de novo protein synthesis (Tigges et al., 1992). Peptide fragments of structural proteins would be the first to enter antigen processing pathways and would be most likely to escape the early blockade imposed by ICP47. These observations support the notion that ICP47 may be a very effective inhibitor of $CD8^+$ T lymphocyte responses in humans, not only of CTLs but also other subsets of $CD8^+$ T lymphocytes that, for example, produce antiviral cytokines. The effects of ICP47 may have important implications in the design of HSV vaccines. Furthermore, we expect that ICP47 may be very useful as a small, mobile immune suppressive agent in virus-based gene therapy vectors, in tissue transplantation, or in the therapy of certain autoimmune diseases.

Experimental Procedures

Cells and Viruses

Vero (African green monkey kidney) cells, MC57G (mouse H-2^b haplotype) cells, Sv/BALB cells (mouse H-$2K^k$ haplotype) (Gooding, 1979), 293 cells (Graham et al., 1977), and normal human fibroblasts derived from skin biopsies (used between passages 10–20) were all passaged in α-minimal essential media (α-MEM) containing 5%–10% fetal bovine serum (FBS). HSV-1 strains F (Ejercito et al., 1968) and KOS; the HSV-1 deletion mutant Vhs2 lacking the virion host shutoff gene *UL41* (Smibert and Smiley, 1990); the HSV-1 ICP0 deletion mutant *dl*x3.1 (Sacks and Schaffer, 1987); the HSV-1 ICP6 deletion virus *hrR3* (Goldstein and Weller, 1988); the HSV-1 ICP22 deletion mutant virus *R325-5.7IC*$^-$ (Sears et al., 1985); the HSV-1 ICP47 mutants *N38* (Umene, 1986) and *R3631* (Mavromara-Nazos et al., 1986); HSV-2 strain 333; and an HSV-2 mutant in which the virion host shutoff coding sequences were disrupted (C. A. Smibert and J. R. Smiley, unpublished data) were all propagated and titered on Vero cells. The HSV-1 ICP4 mutant *d*120 was propagated on E5 cells (DeLuca et al., 1985), and the HSV-1 ICP27 deletion mutant virus *5dl*1.2 was propagated on 3-3 cells (McCarthy et al., 1989). The HSV-1 gD$^-$ mutant *F-US6KAN* was grown on VD60 cells (Ligas and Johnson, 1988). HCMV strain Ad169 was propagated and titered on normal human fibroblasts.

Construction of Recombinant Viruses

Recombinant HSV, F-US5MHC, and F-US5S were constructed by rescue of the gD$^-$ mutant HSV-1 *F-US6KAN* with plasmids containing either the MHC genes derived from pcKb (Schönrich et al., 1991) and pVcβ2 (Daniel et al., 1983) or the *lacZ* gene from pD6p (Goldstein and Weller, 1988), as previously described (Feenstra et al., 1990). An adenovirus, AdICP47-1, expressing ICP47 was constructed by rescue of a plasmid derived from pCA4 (C. Addison and F. L. G., unpublished data), which contains the left end (16%) of the Ad5 genome with a deletion of the E1 region into which is inserted the HCMV immediate-early promoter, a polylinker, and the SV40 polyadenylation signal, with plasmid pJM17 (McGrory et al., 1988) in 293 cells, producing recombinant AdICP47-1, which was plaque purified and propagated on 293 cells.

Antibodies and Peptides

The MAbs W6/32 (Parham et al., 1979) and Y3 (Jones and Janeway, 1981) were obtained from ATCC. Rabbit antiserum specific for mouse MHC class I heavy chain H-$2K^k$ (Smith et al., 1986) was provided by B. Barber (University of Toronto, Toronto, Ontario, Canada). HC10 (Stam et al., 1986) was provided by H. Ploegh (Massachussetts Institute of Technology, Cambridge, Massachusetts). MA2.1 (Weys et al., 1986) was provided by T. Spies (Dana–Farber Cancer Institute, Boston, Massachusetts). A rabbit antiserum raised to human β_2m, which cross-reacts with mouse β_2m, was obtained from Dakopatts (Copenhagen, Denmark). A rabbit polyclonal antiserum directed against a peptide corresponding to the C-terminus of ICP47 (Paltreyman et al., 1984) was obtained from H. Marsden (Institute on Virology, Glasgow, Scotland) and was later produced at McMaster University. LP2, a MAb specific for HSV gD (Minson et al., 1986), was a gift of A. C. Minson (Cambridge University, Cambridge, England). T56/14, a MAb specific for the transferrin receptor, was obtained from Oncogene Science (Uniondale, New York).

Peptides corresponding to residues 478–484 of HIV reverse transcriptase (ILKEPVHGV), which binds to HLA-A2 (Walker et al., 1989), and gB498-505 (Bonneau et al., 1993), which binds to H-$2K^k$, were synthesized and purified by HPLC in the McMaster Molecular Biology and Biotechnology Institute.

Radiolabeling of Cells, Immunoprecipitations, Stability Assays, Endo H Digestions, and Gel Electrophoresis

The stability of the MHC class I protein complex was determined by labeling uninfected or HSV-infected fibroblasts for 1 hr with [^{35}S]methionine and [^{35}S]cysteine. Cells were lysed with 1% NP-40, 0.5% DOC buffer containing 5 mg/ml BSA, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, and 0.1 TIU/ml aprotinin and mixed with the appropriate peptide and then incubated for 18 hr at 4°C. MHC class I was immunoprecipitated directly with MA2.1 as previously described (Johnson and Feenstra, 1987) or extracts were first heated at 70°C for 1 hr to denature MHC class I molecules partially before precipitation with HC10. Pulse-chase experiments were performed by labeling cells for 20 or 30 min. The cells were lysed immediately or washed and incubated in α-MEM containing 1% FBS for 90 min before lysis (chase). Endo H digestions were performed according to the recomendations of the manufacturer (New England Biolabs, Mississauga, Ontario, Canada).

In Vitro Translation with Microsomes

Microsomal and mitochondrial membrane fractions were derived from human KB cells as described previously (Atkinson and Summers, 1971; Janiak et al., 1994a). ER membranes were pelleted and suspended in in 50 mM triethanolamine (pH 7.5), 1 mM $MgCl_2$, 0.5 mM EDTA, 1.0 mM DTT. The ICP47 open reading frame was amplified by PCR from plasmid pRHP6 and inserted into pSPUTK to produce plasmid pSPUTK-47. Plasmids pSPUTK-47, pSPUTK-gPA (Janiak et al., 1994a), and pSPUTK-Cβ5 (Janiak et al., 1994b) were transcribed using SP6 polymerase, and transcription products were added directly to reticulocyte lysate translation reactions and then incubated with membrane fractions and subsequently analyzed by gel filtration as described previously (Janiak et al., 1994a).

Immunofluorescence Experiments

Human fibroblasts were infected with AdICP47-1 or AddlE1 using 150 pfu/cell for 24 hr or 36 hr or were left uninfected and then fixed with 4% paraformaldehyde for 15 min, washed with PBS, and permeabilized with 0.2% Triton X-100 for 5 min. The cells were washed, incubated with 0.2 M ethanolamine (pH 7.0) for 1 hr, washed, and then incubated for 1 hr with PBS containing 2% BSA (PBS–BSA) and 2% goat serum. Fibroblasts were incubated with rabbit anti-ICP47 antibodies, diluted 1:200 in PBS–BSA and extensively adsorbed against fixed, permeabilized fibroblasts, and simultaneously with mouse anti-HLA MAbs W6/32, MB40.5, and BB7.5 (ascites fluids diluted 1:200 in PBS–BSA) for 1 hr at room temperature. The cells were washed with PBS–BSA, incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) antibodies and rhodamine-conjugated goat anti-mouse IgG antibodies for 45 min and then washed and mounted on microscope slides.

^{51}Cr Release Assays

Mouse anti-HSV CTLs were generated as previously described (Pfizenmaier et al., 1977; Hanke et al., 1991). Mouse or human target cells were infected with HSV-1, using 10 pfu/cell for 2–4 hr, and then loaded with ^{51}Cr for 1–2 hr and mixed with mouse CTLs for 4–5 hr. The human CTL clones MR-16E6 ($CD3^+CD8^+$ HLA-B35 restricted and specific for an epitope derived from HCMV pp65) and DG-28D3 ($CD3^+CD8^+$ HLA-B14 restricted and specific for an unknown HCMV protein) were isolated and grown as described previously (Riddell and Greenberg, 1990; Riddell et al., 1991). Human fibroblasts derived from donor MR (A24, A25, B18, B35, DR3) or fibroblasts from donor DG (A69, B14) were left uninfected or infected with AdICP47 or AddlE1 using 100 pfu/cell for 24 hr and then infected with HCMV (AD169 strain) using 5 pfu/ml for 12 hr. These target cells were labeled with ^{51}Cr for 2 hr and then tested in a ^{51}Cr release assay with appropriate T cell effectors for 5 hr (Hanke et al., 1991).

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CHAPTER 2 DISCUSSION

This chapter describes a mechanism by which HSV evades $CD8^+$ CTL recognition, and the protein responsible for the effect, ICP47. After this work was in press, the same phenomenon was described by Hill and colleagues (1994), although the effect was ascribed to an early rather than an immediate-early gene. As with Chapter 1, the questions of the biological relevance and the mechanism of action must be addressed. However, in this case, the answers seem more clear.

It seems likely that this phenomenon is relevant to HSV pathogenesis. The best evidence for this may be the unusually high proportion of $CD4^+$ CTL specific for HSV in humans, but not in mice. Since it appears that ICP47 has little effect on MHC class I transport in mouse cells, mice should, and apparently do, generate HSV-specific $CD8^+$ CTL efficiently, whereas in the absence of MHC class I charged with HSV-specific peptides, human CTL will not be efficiently amplified. Other evidence for the importance of the ICP47 effect is discussed in Chapter 2.

To date, the mechanism of action of ICP47 is obvious to a point, and impenetrable beyond that point. MHC class I in the presence of ICP47 lacks associated peptide. Clearly, ICP47 somehow blocks the assembly of the mature MHC class I complex. Since ICP47 appears to be a cytoplasmic protein, and does not associate with membranes, it is less likely to impinge on steps of the antigen processing pathway inside the ER, or at the ER surface. Cytoplasmic steps in antigen processing include targeting of proteins to the proteasome for proteolysis, proteasome-mediated proteolysis, and transport of peptides to the TAP complex at the ER surface. All of these are potential sites for ICP47 action.

Proteins destined to be degraded by the proteasome are covalently tagged with an 8.5 kDa protein, ubiquitin (Matthews et al., 1989; Seufert and Jentsch, 1992). The steps involved in ubiquitination are complex, involving several enzymes (reviewed in Finley, 1991; Hershko and Ciechanover, 1992; Jentsch, 1992), including some redundant enzymes. Since temperature-sensitive mutants in a ubiquitin-conjugating enzyme are defective in antigen presentation at the non-permissive temperature (Michalek et et al., 1993), this pathway is a potential target for ICP47.

It is worth noting that enhancement of ubiquitin-conjugating activity could conceivably inhibit antigen presentation, whether by increased production of a ubiquitinated inhibitor of proteasome activity (Li and Etlinger, 1992), or by altering turnover of other components of the antigen presentation pathway (for example, Sommer and Jentsch, 1993).

Either inhibition or enhancement of proteasome activity could prevent antigen presentation. Inhibition of proteasome activity would prevent the production of antigenic peptides from proteins, whereas enhancement of activity could cause the proteins to be degraded past the eight-amino acid minimum length for MHC class I binding. The multisubunit nature of the proteasome could make it more vulnerable to alterations (by providing multiple potential targets) or might make it resistant (by redundancy either at the level of proteasome subunits, or at the level of proteasome subclasses.)

Both ubiquitination and (probably) proteasome function are essential for cell viability, and complete inhibition by ICP47 would not be compatible with long term cell survival. Cells are viable for many days after infection with AdICP47-1, which might argue that neither of these is the target for ICP47.

However, careful evaluation of long-term viability of cells expressing ICP47 has not yet been performed, so the possibility remains open.

After peptide generation, the next well-defined step in antigen presentation is transport across the ER membrane by the TAP complex. There is, however, circumstantial evidence that another intracellular step may be involved in antigen processing: transport from the proteasome to the TAP complex by heat-shock proteins (reviewed in Srivastava et al., 1994.) If this step is blocked by ICP47, then destruction of peptides in the cytoplasm might overbalance TAP-mediated transport, so that an adequate concentration of peptide would not be reached in the ER.

Finally, although a direct interaction with the TAP transporter, MHC class I, or other post-ER components of the antigen presentation pathway seems unlikely in light of the cytoplasmic location of ICP47, it is still possible that ICP47 alters the cytoplasmic domain of one of these proteins, or affects one of these events indirectly. Also, the current understanding of MHC class I antigen processing has been gained mainly from mutant cell lines lacking TAP activity, all of which are lymphoblastoid cells: ICP47 appears to affect lymphoblastoid cells less efficiently than other cells types such as fibroblasts. It is possible that in other cell types more, or different, steps are essential for antigen processing, and it is one of these steps that is affected by ICP47.

A number of experiments could help address the mechanism of action of ICP47. It would be useful to further define the stage which is inhibited by ICP47. If the lesion is in the generation of antigenic peptides, then the defect in antigen presentation could be overcome by expressing an appropriate peptide as a minigene or by supplying peptides to a permeabilized cell. If, however, the

defect is at the level of transport across the ER membrane, then expressing the peptide as a minigene will not overcome the the failure of recognition; however, expression of the peptide fused to a signal sequence may overcome this by recruiting the normal signal recognition particle to cross the ER membrane.

Known components of the antigen processing pathway, such as the proteasome, and the TAP transporters, could be examined for altered expression in HSV-infected or other ICP47-expressing cells, and could also be examined for interactions with ICP47. Such experiments are in progress, but have not as yet had clear results.

Since ICP47 presumably interacts with some cellular component, if only transiently, the standard procedures for looking for protein-protein interactions could be used. Ligand blotting, co-immunoprecipitation, chemical cross-linking, and the two-hybrid system in yeast, are all possible approaches, and experiments involving these techniques are in progress. The most encouraging results so far have involved precipitation of cellular extracts with a glutathione S-transferase/ICP47 fusion protein, and is discussed in the next chapter.

One striking finding with regard to HSV-induced inhibition of MHC class I transport is that not all human cells appear to be affected. Thus, normal fibroblasts (the subject of most of the studies described above), HeLa cells, and KB cells all show a clear inhibition in MHC class I transport after HSV infection. In contrast, human lymphoblastoid cell lines (LCL) infected with HSV are lysed effectively by HSV-specific $CD8^+$ CTL (Yasukawa et al., 1989; Posavad and Rosenthal, 1992; Posavad et al., 1993), and preliminary experiments suggest that some other human cell types are also resistant to the effects of ICP47. However, it is not known whether the resistance is absolute, or if it could be overcome by

increasing levels of expression of ICP47; nor is it clear whether the effect is truly tissue-specific or an artifact of transformation. Furthermore, the $CD8^+$ CTL used to lyse LCL are generally directed against structural proteins (Tigges et al., 1992), which are the most likely to avoid the effects of ICP47. Nevertheless, if some cell types are indeed completely resistant to ICP47, it is possible that MHC class I antigen processing involves different steps in different cell types, which in turn has intriguing implications for the cell biology of antigen presentation.

The purpose of this thesis was to define mechanisms by which HSV evades the cellular immune system. Chapter 2 shows that HSV can avoid recognition by $CD8^+$ CTL by blocking antigen processing in some cell types. The mechanism by which antigen processing is blocked is not known. The next chapter shows that a cellular protein binds specifically to ICP47, and therefore may help shed light on the mechanism of ICP47.

CHAPTER 3

CHAPTER 3. HERPES SIMPLEX VIRUS ICP47 BINDS TO AN 8.5 kDa CELLULAR PROTEIN

Introduction.

The herpes simplex virus immediate-early protein ICP47 prevents CD8+ lymphocytes from lysing target cells, due to a blockade of MHC class I antigen processing. Expression of ICP47 in susceptible cells results in the retention of MHC class I in the ER, and newly synthesized MHC class I in these cells is less stable than in normal cells. These phenomena have been associated with a lack of peptide associated with the MHC class I heavy chain and β_2-m. The effect of ICP47, therefore, may be due to a failure to produce peptide, a failure to properly transport the peptide to and across the ER membrane, or a defect in assembly of the mature MHC class I trimolecular complex. Searches of the Genbank, Swissprot, and EMBL protein libraries using the BLASTP, BLITZ, and FASTA programs did not find any proteins with significant homology to ICP47, and examination of ICP47 for known protein motifs using the PROSITE databanks did not reveal any functional motifs. The mode of action of ICP47 is not yet known.

Antigenic peptide associated with MHC class I is predominantly produced by proteolytic degradation in the cytoplasm. The machinery involved in this degradation is not yet completely clear; however, the 26S proteasome complex is the best candidate. In most cases, the evidence for proteasome involvement in antigen processing is circumstantial. At least one antigen (ovalbumin) is not presented to class I-restricted T cells in cells temperature sensitive (ts) for ubiquitin function, at the non-permissive temperature, although presentation is efficient at the permissive temperature (Michalek et al., 1993). (Ubiquitination of proteins results in their targeting to the proteasome for proteolysis.) In vitro

examination of proteasome function has shown that peptides produced by proteasomes containing LMP2 and LMP7 (proteasome subunits encoded in the MHC class II region) have basic or hydrophobic residues at their carboxy terminus (Driscoll et al., 1993; Gaczynska et al., 1993), which is consistent with the carboxy-terminal residues of MHC class I-binding peptides (Falk and Rotzschke, 1993). There is also evidence, however, that other proteases can be involved in antigen presentation pathways (Eisenlohr et al., 1992).

Since peptides are produced in the cytoplasm and associate with MHC class I in the ER, the peptides must cross the ER membrane. Although in some cases peptides are able to cross the membrane non-specifically (Wei and Cresswell, 1992; Zhou et al., 1993a, b), in most cases a heterodimer of TAP1 and TAP2, transmembrane proteins which are members of the ABC family of transport proteins, is required for efficient transport (Androlewicz et al., 1993; Neefjes et al., 1993; Momburg et al., 1994). The ABC family of transport proteins is a large class of bacterial and eukaryotic proteins with an ATP-binding cassette in the cytoplasmic domain and either 6 or 12 membrane-spanning regions: proteins with 6 membrane-spanning regions act as homo- or heterodimers. These proteins transport substances ranging from ions to large proteins across membranes.

Sequence analysis of ICP47 does not reveal any potential transmembrane regions or signal or targeting sequences. Previous work (Palfreyman et al., 1984; York et al., 1994), shows that ICP47 is not membrane-associated. Potential targets for ICP47 include cytoplasmic components of the antigen presentation pathway. However, since cytoplasmic events in MHC class I antigen processing are not yet well understood, another approach to understanding the mechanism of action of the ICP47 is to identify cellular proteins with which it interacts.

Materials and Methods.

Cells and virus. Cells used in these experiments were human normal fibroblasts, used between passage 6 and 20, obtained from K. Rosenthal, McMaster University; normal mouse fibroblasts, derived from either C57BL/6 or BALB/c mice, used between passage 10 and 20; IMR-32 (human neuroblastoma cells), HeLa cells (human cervical epidermoid carcinoma cells) (Gey et al., 1952), and KB cells (human oral epidermoid carcinoma) (Eagle, 1955), obtained from ATCC; all of which were grown in αMEM supplemented with 10% fetal bovine serum (FBS), glutamine, and penicillin/streptomycin (P/S); 293 cells (adenovirus type 5-transformed human kidney epithelial cells) (Graham et al., 1977), obtained from F. Graham (McMaster University), grown in F11 medium/10% FBS/glutamine/P/S; B6/WT-3 (mouse SV40-transformed fibroblasts) (Pretell et al., 1979), obtained from S. Tevethia (University of Pennsylvania, Hershey, PA); R970-5 (human osteosarcoma cells) (Rhim et al., 1975), obtained from K. Huebner and C. Croce (Wistar Inst., Philadelphia, PA); and Vero (African Green Monkey kidney cells), were grown in αMEM/5% FBS/glutamine/P/S; human EBV-transformed B lymphocyte cell lines (LCL), obtained from K. Rosenthal (McMaster University); and K562 (human myelogenous leukemia cells) (Lozzio and Lozzio, 1975), obtained from ATCC; which were grown in RPMI medium/10% FBS/glutamine/P/S/HEPES, further supplemented with 1 x 10^{-5} M β-mercaptoethanol in the case of LCL; and High Five cells (cabbage looper moth egg cells) (Invitrogen, San Diego, CA)), grown in ExCell medium (Invitrogen) supplemented with glutamine/P/S.

Antibodies. Antibodies used in these experiments were C47-1 (rabbit antiserum directed against the carboxy terminal peptide of HSV-1 ICP47) (Palfreyman et al., 1984; York et al., 1994) and rabbit anti-ubiquitin antiserum (Sigma Chemical Co., St. Louis, MO).

Overexpression and purification of a bacterial GST-ICP47 fusion protein. Plasmid p47EX was constructed by inserting the EcoRI - XhoI fragment from pRHP6 (Persson et al., 1985) into the EcoRI - SalI region of pUC19. The polymerase chain reaction (PCR), using primers AB3014 (5'-TAGGATCCCA TATGTCGTGG GCCCTGGAAAT-3') and AB3015 (5'-TAGAATTCGA CTCGGGTGAT GGTCGTA-3'), was used to modify sequences from the plasmid p47EX so that ICP47 sequences were immediately flanked by BamHI and NdeI (5') and EcoRI (3') restriction sites. The product of PCR was ligated into pUC19 to produce plasmid p47BE. The BamHI - EcoRI fragment containing ICP47 sequences were ligated into the BamHI - EcoRI region of pGEX-2T (Pharmacia LKB Biotechnology, Baie d'Urfé, Quebec), to produce a plasmid, pGEX-2T47, encoding a fusion protein consisting of glutathione S-transferase fused at its carboxy terminus to full length ICP47, with a thrombin cleavage site at the junction. The BamHI - EcoRI fragment was also inserted into the BamHI-EcoRI region of pGEX-3X to produce pGEX-3X47X, which encodes an out-of-frame fusion protein. pGEX-3X47X was cut with BamHI, digested with mung bean nuclease, and religated in order to produce pGEX-3X47, which encodes an in-frame fusion protein which can be cleaved by factor Xa rather than by thrombin.

E. coli (DH5α strain) were transformed with pGEX-2T, pGEX-2T47, pGEX-3X, or pGEX-3X47, grown in Luria Broth to OD_{595} 0.6 - 0.9, and induced with 1 mM IPTG for 2 hr at 37°C. The bacteria were pelleted and lysed with a French Press, the clarified lysate was applied to a Sepharose-glutathione column (Pharmacia LKB Biotechnology), washed with phosphate-buffered saline (PBS)/1% Triton X-100, and eluted with 5 mM free glutathione. In some cases, GST-2T47 was cleaved with thrombin (Sigma) to produce free ICP47. To produce ICP47-Sepharose, free ICP47 was covalently coupled to Sepharose 4B (Pharmacia) by the cyanogen bromide method (Axén et al., 1967).

Subcellular fractionation. Approximately 5 x 10^6 cells were metabolically labelled with ^{35}S-methionine (100 μCi/ml) for 2 hours. For subcellular fractionation, cells were washed with phosphate-buffered saline (PBS), and suspended in 10 mM Tris, pH 7.5/1.5 mM $MgCl_2$ (S-100 buffer)/1mM PMSF. After 10 min incubation on ice, the cells were disrupted in a dounce homogenizer (25 strokes, A pestle). The sample was centrifuged at 3000 RPM in an SS34 rotor at 4° C for 20 min. The pellet obtained from this spin (nuclear fraction) was resuspended in S-100 buffer/0.5% octylglucoside/2 mg/ml bovine serum albumin/200 mM NaCl/1mM PMSF (S-100/OG/NaCl/BSA) and the supernatant liquid was centrifuged at 100,000 g for 30 min at 4°C. The pellet obtained from this spin was resuspended in S-100/OG/NaCl/BSA as the membrane fraction, and the supernatant liquid was used as the cytoplasmic fraction.

Precipitation of cellular proteins. Metabolically labelled cells were lysed in S-100/OG/BSA. In some cases, NaCl was added to 100 - 1000 mM. To the cellular

lysate, or to the samples obtained by subcellular fractionation, was added 5 μl of either GST-2T, GST-3X, GST-2T47 or GST-3X47 bound to glutathione-Sepharose, or 10 μl of ICP47-Sepharose or IgG-Sepharose (Pharmacia). Samples were incubated end-over-end at 4°C for 2 hr, washed 3 times with S-100/OG, and the washed pellet was boiled with 10 μl of 2x Laemmli's loading buffer. Samples were separated by 15% DATD-polyacrylamide gel electrophoresis, fixed, treated with Enlightening (NEN Research Products, Boston, MA), dried, and exposed to film.

Immunoprecipitations were performed as previously described (Johnson and Feenstra, 1987).

Results.

ICP47 binds to an 8.5 kDa cytoplasmic protein. Bacteria transformed with pGEX-2T47 expressed a glutathione-Sepharose-binding protein of approximately 32 kDa on induction with IPTG (GST-2T47); bacteria transformed with pGEX-2T alone expressed a glutathione-Sepharose-binding protein of approximately 22 kDa (Figure 1; pp. 51-52). Since ICP47 is approximately 10 kDa, the 32 kDa protein is the appropriate size for the GST-ICP47 fusion protein. Furthermore, on treatment with thrombin, the 32 kDa protein was cleaved to 21 and 10 kDa fragments (Figure 1; pp. 51-52)

Glutathione-Sepharose beads bound to GST or to GST-47, or free ICP47 covalently coupled to Sepharose, were used to precipitate cellular proteins. GST-47, either derived from pGEX-2T47 or pGEX-3X47 (not shown) specifically bound to a protein (ICP47 binding protein: 47BP) from normal human fibroblasts, of approximately 8.5 - 9.0 kDa, which did not bind to GST alone (Figure 2; pp.

53-54). A protein of identical size bound to ICP47-Sepharose, but not to IgG Sepharose (Figure 2 pp. 53-54). This protein remained bound to GST-47 and to ICP47-Sepharose in the presence of 200 and 500 mM NaCl. Subcellular fractionation suggested that 47BP was present in cytoplasmic and nuclear fractions; no 47BP was found in membrane fractions (Figure 3; pp. 55-56). Therefore, 47BP appears to be in the same subcellular compartments as ICP47 (York et al., 1994).

A number of cell types were tested for the presence of 47BP. As well as normal human fibroblasts, 47BP was specifically precipitated by GST-ICP47 from KB, B6/WT-3, R970, HeLa, and IMR-32 cells (Figure 4; pp. 57-58), as well as primary fibroblasts derived from C57BL/6 mice, 143B, Vero, COS-1, and SVBALB cells (not shown). No proteins were specifically precipitated by GST-47 from lysates of 293, LCL, K562, or High Five cells (Figure 4; pp. 57-58).

The size of the protein suggested that it might be ubiquitin, a ubiquitous protein of approximately 8.5 kDa involved in cytoplasmic proteolysis and implicated in antigen processing (Michalek et al., 1993). Comparison of immunoprecipitated ubiquitin to 47BP showed that uibiquitin migrates slightly faster than does 47BP on DATD-PAGE (Figure 5; pp. 59-60).

Discussion.

The HSV IE protein ICP47 blocks antigen processing at some stage prior to assembly of the mature MHC class I complex, and appears to act in the cytoplasm. Here I describe a cellular protein which appears to bind specifically to ICP47, and which is therefore a possible target for ICP47 action.

The cellular protein (47BP) has a molecular mass of approximately 8.5 kDa and is found in the cytoplasm and nucleus but not in the membrane fraction of cells - the same distribution as ICP47. It was originally identified as binding to a fusion protein consisting of ICP47 fused to the carboxy terminus of GST, with a junction sequence containing a thrombin cleavage site; it also binds to a GST-ICP47 fusion protein containing a factor Xa cleavage site (not shown), as well as to ICP47 cleaved from GST-2T47 and covalently bound to Sepharose. Binding was specific, as GST-2T and GST-3X the GST products from the parent vectors pGEX-2T and pGEX-3X respectively, which contain the thrombin or Factor Xa cleavage site respectively fused to the carboxy terminus of GST), and IgG coupled to Sepharose, did not bind detectable amounts of 47BP. Binding was not inhibited by physiological and greater levels of salt (Figure 2), but was less efficient in the presence of some detergents, such as NP40 (not shown). Occasionally an additional, slightly larger protein was also seen (e.g. Figure 3, nuclear fraction). This was seen most consistently in HeLa cell extracts (Figure 4: in other HeLa extracts the bands were of approximately equal intensity), but appeared inconsistently in fibroblast lysates as well.

47BP was found in several cell types: fibroblasts (both human and mouse), human cervical carcinoma (HeLa, KB), human osteosarcoma (R970, 143B), human neuroblastoma (IMR-32), and monkey kidney epithelium (Vero, COS-1); but not in a cell line derived from human kidney epithelium (293), or lymphoid cells (EBV-transformed B lymphocytes, K562) or in an insect cell line (High 5). There is no obvious common theme in this pattern. However, it is worth noting that, in general, the expression of 47BP correlates with the sensitivity of the cell type to ICP47's effect on antigen processing. Human fibroblasts, which are clearly

susceptible to ICP47, expressed large amounts of 47BP, while LCL, which remain sensitive to MHC class I-restricted, HSV-specific CTL lysis after infection with HSV (Posavad and Rosenthal, 1992; Posavad et al., 1993; Koelle et al., 1993) did not express detectable amounts of 47BP. Preliminary experiments indicate that several other cell types which express 47BP (HeLa, KB, COS, R970, Vero, and mouse primary fibroblasts) are at least partially sensitive to ICP47. On the other hand, preliminary experiments also suggest that IMR-32 cells, which express 47BP, are not fully sensitive to ICP47. Hence, while 47BP is a reasonable candidate for a cellular target for ICP47, a better understanding of the mechanism by which ICP47 acts will be necessary to clarify the role of 47BP in antigen processing.

Since 47BP is cytoplasmic, it could be involved in limiting or enhancing proteolysis; it could help transport peptides to the TAP transporter complex, or stabilize them; or it could have a role in the transport of peptides across the ER membrane. For example, 47BP could provide ATP for the TAP transporters, since the ATP-binding cassette in these proteins is on the cytoplasmic face of the membrane (Bahram et al., 1991). The absence of 47BP in some cell types, such as LCL, which are capable of antigen presentation, may suggest that 47BP is not required for antigen processing in these cells. 47BP could be a member of a family of proteins with tissue-specific expression, or it may be present in some cells but not available for binding with ICP47 - for example, tightly bound in a complex. Until 47BP is purified in quantities large enough for N-terminal peptide sequencing, these speculations are not likely to be resolved.

Figure 1. Expression of GST-ICP47 fusion proteins. *E. coli* (DH5α strain) were transformed with pGEX-2T47 (GST-47) or pGEX-2T (GST) and induced with 1 mM IPTG. Bacterial pellets were lysed and clarified and applied to glutathione-Sepharose columns. After washing, protein was eluted with free glutathione. In some cases, after binding to the column, protein was digested with 1/1000 or 1/5000 the amount of thrombin. After electrophoresis on a 15% DATD-polyacrylamide gel was stained with Coomassie Blue.

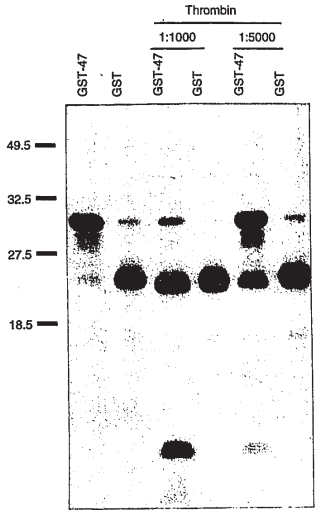


Figure 2. ICP47 binds a cellular protein of approximately 8.5 kDa. Human normal fibroblasts were radiolabelled and lysed in 10 mM Tris pH 7.5/1.5 mM $MgCl_2$/0.5% octylglucoside/2 mg/ml BSA/1 mM PMSF. Some samples were adjusted to 500 mM or 200 mM NaCl. After centrifugation, the samples were precipitated with IgG coupled to sepharose (0 mM NaCl); with ICP47 coupled to Sepharose (0 mM and 500 mM NaCl); or with GST-2T or GST-2T47 bound to glutathione-Sepharose (200 mM NaCl). After washing, samples were electrophoresed on 15% DATD-polyacrylamide and autoradiographed.

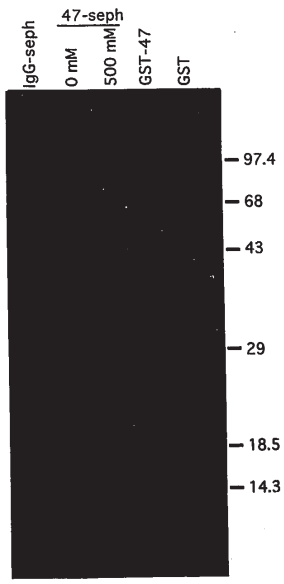


Figure 3. The cellular protein which binds to ICP47 is not membrane-associated. Human primary fibroblasts were metabolically labelled with ^{35}S-methionine for 2 hr, separated into cytoplasmic, nuclear, and membrane fractions as described in Materials and Methods, or lysed in 10 mM Tris pH 7.5/1.5 mM $MgCl_2$/0.5% octylglucoside/200 mM NaCl/2 mg/ml BSA/1 mM PMSF (Total), and samples were precipitated with GST-2T or GST-2T47 as described in Materials and Methods.

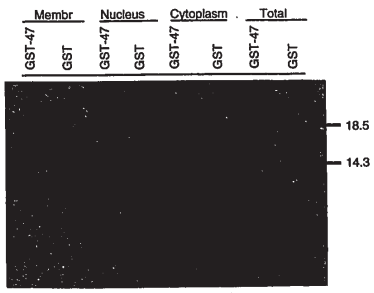
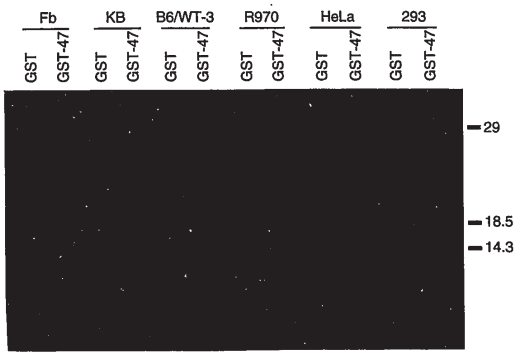


Figure 4. Expression of the cellular protein which binds to ICP47 is cell-type dependent. Human fibroblasts (Fb), human cervical carcinoma cells (KB), mouse SV40-transformed fibroblasts (B6/WT-3), human osteosarcoma cells (R970), human cevical carcinoma cells (HeLa), human kidney cells transformed with the E1 region of adenovirus type 5 (293), human neuroblastoma cells (IMR-32), human EBV-transformed lymphoblastoid cell lines (LCL), human myelogenous leukemia cells (K562), and an insect cell line (High 5), were radiolabelled, lysed with 10 mM Tris pH 7.5/1.5 mM $MgCl_2$/0.5% octylglucoside/2 mg/ml BSA/1 mM PMSF, and precipitated with GST-2T or GST-2T47 as described in Materials and Methods.



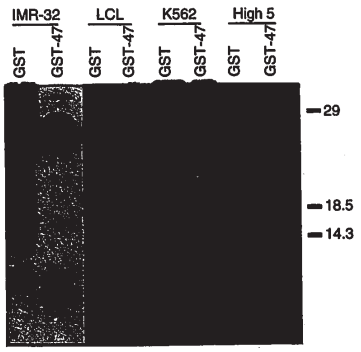
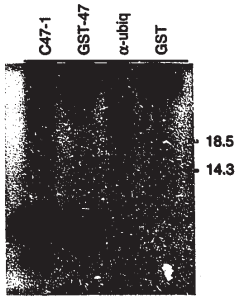


Figure 5. The cellular protein which binds to ICP47 is not ubiquitin. Human normal fibroblasts were infected for 2 hours with HSV-1(F), metabolically labelled and lysed as described in the legend to Figure 4, and the lysate was precipitated with GST or GST-47 as described in the Materials and Methods, or immunoprecipitated with a rabbit antiserum directed against the carboxy terminus of ICP47 (C47-1) or with a rabbit antiserum to ubiquitin (α-ubiq).



DISCUSSION

One of the most intriguing aspects of HSV infection in humans is its ability to reactivate and cause recrudescent infection, in spite of an ostensibly competent immune system. The usual explanation for this ability is that "stress" or other causes of immune suppression are responsible; the implication is that the virus plays a passive role, opportunistically taking advantage of a weakened host. The observations described in this thesis suggest that the virus takes a more active role in recurrent infection: while modest immune suppression probably enhances the reactivation process, HSV is quite capable of coping with many aspects of the immune system.

The two forms of immune evasion described above (inhibition of LAK cells by cell-to-cell spread from infected target cells to effector cell, and ICP47-mediated inhibition of MHC class I antigen presentation) are both likely to be important in natural infection of humans. Neither, however, are likely to be of great importance in infection of mice, as neither the inhibition of LAK cells nor the blockade of antigen presentation is particularly efficient in mouse cells.. Until more is known about the mechanism involved, the difference between human and mouse cells is unlikely to be clarified.

Since HSV evades the human immune system more efficiently than the mouse immune system, the mouse may not be an ideal model for anti-HSV immunity. Studies involving predominantly humoral immunity are probably, in general, valid (although the HSV Fc receptor does not bind to mouse IgG, in contrast to rabbit for HSV-1 and human IgG); but those studies based on cellular immunity may need to be reevaluated. It is interesting, however, that mouse models suggest indirectly that ICP47 may play a role in herpetic keratitis. It has

DISCUSSION

been observed that, in mice, keratitis is most severe when the lymphocyte response is primarily of the $CD4^+$ phenotype, while the presence of $CD8^+$ lymphocytes appears to be protective (reviewed in Hendricks and Tumpey, 1990). Since ICP47 expression would bias the lymphocyte response toward a $CD4^+$-dominated response, it is possible that ICP47 may be a useful target for therapy of herpetic keratitis. Preliminary experiments in collaboration with Robert Hendricks (University of Chicago) suggest that this might be the case.

ICP47 may also have clinical value in situations in which $CD8^+$ CTL responses are harmful. Such situations might include autoimmune disease, transplantation, and gene therapy. The approach would be to deliver ICP47 to the target tissue, perhaps as a component of a gene therapy vector or in the form of an expression vector. The addition of ICP47 to a gene therapy vector should be relatively straightforward; the gene has the advantage of being small, so it will not interfere significantly with the carrying capacity of the vector. In mouse livers, adenovirus vectors are eliminated by a CTL response (Yang et al., 1994) It is conceivable, then, that ICP47 may prolong the effective period of gene therapy vectors.

$ß_2$-microglobulin-knockout mice have been used as donors for transplantation of several tissues. Although bone marrow transplants were rejected as rapidly as those from wild-type mice, skin transplants (Zijlstra et al., 1992) and pancreatic islet transplants (Osorio et al., 1993) were rejected much less efficiently. This implies that, at least in certain forms of transplantation, rejection is predominantly mediated by $CD8^+$ CTL, and that therefore expression of ICP47 in tissues may help engraftment. In the case of skin transplants, in

particular, efficient delivery of ICP47 to the donor tissue may be less of a problem, since the tissue is relatively accessible for manipulation.

Another interesting possibility is that ICP47 is responsible for the increased sensitivity to NK cell lysis observed in HSV-infected cells. The gene(s) responsible for this sensitivity have been identified as immediate-early, based on mutant viruses and drug blocks (Fitzgerald-Bocarsly et al., 1991). NK cells have been shown to specifically lyse cells with low cell surface expression of MHC class I; however, HSV-infected cells are lysed before ICP47 would be expected to significantly alter cell-surface expression of MHC class I. The mechanism by which NK cells recognize low MHC class I expression is not known. ICP47 provides an opportunity to examine this question: shortly after ICP47 is expressed, overall cell surface levels of MHC class I will not be greatly changed, but newly synthesized MHC class I complexes will be empty of peptide. If cells at this time show increased NK sensitivity, a role for empty MHC class I molecules will be more likely.

It is somewhat surprising that no homologues for ICP47 have been identified. Even the related α–herpesviruses pseudorabies and varicella-zoster virus apparently have no related protein (Davison and McGeoch, 1986; Zhang and Leader, 1990). It is possible that a small open reading frame with low homology may have been missed; it is also possible that laboratory strains of virus which had lost expression of ICP47 homologues were used for the sequencing. Examination of the more closely related viruses bovine herpesvirus 2 and simian agent 8 (McGeoch and Cook, 1994) may prove fruitful. However, it is noteworthy that two other herpesviruses, human and murine cytomegalovirus, downregulate MHC class I by mechanisms which differ not only from HSV, but

from each other. It seems likely that most, if not all, herpesviruses have some mechanism for evasion of the antiviral immune response; perhaps antigen presentation pathways are complex enough that each virus can find its own unique method of subverting them.

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APPENDIX: ANTIBODY SPECIFICITY

The following antibodies were used in this thesis.

Chapter 1:
Rabbit anti-HSV antiserum (Dako patts, Copenhagen), specific for HSV structural and probably some non-structural proteins

Chapter 2:
HC10 (monoclonal antibody) specific for MHC class I heavy chain. The antibody was raised against denatured HLA-B and cross-reacts with HLA-C and some HLA-A haplotypes. It reacts poorly or not at all with heavy chain that is associated with ß2-m. Before reacting with this antibody, cell lysates were heated to 75°C for 1 hr, to denature the MHC class I complex and increase the fraction of heavy chain reactive with HC10. HC10 was used in Figure 2A, Figure 3A, andFigure 5B.
LP2 (monoclonal antibody), specific for HSV-1 or -2 gD, was used in Figure 2B.
T56/14 (monoclonal antibody), specific for the transferrin receptor (Oncogene Science, Uniondale, NY), was used in Figure 2C.
MA2.1 (monoclonal antibody), specific for HLA-A2 in association with ß2-m., was used in Figure 3A.
W6/32 (monoclonal antibody), specific for HLA-A,B,C heavy chain/ß2-m complex, was used in Figures 4 and 7. In all cases, the pattern of endoH sensitivity was the same whether HC10 or W6/32 was used.
A mixture of W6/32, MB40.5, and BB7.5 (monoclonal antibodies), each specific for HLA-A,B,C heavy chain/ß2-m complex, was used in Figure 7.
α-C47 (rabbit antiserum) specific for HSV-1 ICP47, raised against a synthetic peptide corresponding to the carboxy terminus of ICP47, was used in Figure 5A.

Chapter 3:
Rabbit antiserum specific for ubiquitin (Sigma Chemical Co., St. Louis, MO) was used in Figure 5.
α-C47 (rabbit antiserum) specific for HSV-1 ICP47, raised against a synthetic peptide corresponding to the carboxy terminus of ICP47, was used in Figure 5.