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Regulation of human leukocyte antigens on cytomegalovirus infected cells

Wohlford, Mark Edward, Ph.D.

The Ohio State University, 1992
REGULATION OF HUMAN LEUKOCYTE ANTIGENS ON CYTOMEGALOVIRUS INFECTED CELLS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By
Mark Edward Wohlford B.S., D.D.S.

* * * * *

The Ohio State University
1992

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DEDICATION

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Wife and Friend
ACKNOWLEDGMENT

Clinicians have long said that their profession involves the art and science of medicine. The dichotomy of "art and science" refer to the learning of medicine by the accumulation of years of clinical experience passed on through generations of practitioners in an apprenticeship system much as that of artists and craftsmen and to the rigorous application of the principles of the scientific method to problems faced in clinical medical practice and the use of modern technological advancements to solve medical dilemmas. Both the clinical artists and the medical scientists have contributed much to the success of modern medicine. In my attempt to become trained both in clinical medicine and science, I have faced many frustrations and rewards. The frustrations made fewer and the rewards greater by the people that I have met along the way and have helped me towards my goals.

Both the clinicians and the scientists have understood the importance of the other half of my training. In retrospect, I am not always sure which category each fit into. Dr. William Wallace provided the financial resources that were essential to this undertaking and Dr. Pete Peterson provided the time in which to accomplish it. My
love and knowledge of science was instilled over many years by many individuals but most recently was provided by Daniel Sedmak who has best blended the art and the science.
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The Major Histocompatibility Complex (MHC) in man is termed the Human Leukocyte Antigen (HLA) system. This system consists of several cell membrane glycoproteins. They are encoded for by a group of linked genes on the short arms of chromosome 6. These genes are some of the most polymorphic genes yet described. The HLA system functions as recognition molecules within the immune system. They bind proteins that are produced either intrinsic or extrinsic to the cell. They present these proteins on the plasma membrane where they are recognized by T-cells as foreign or self. Foreign proteins are identified and then elicit a classic immune response. This response consists of a proliferation of specific T-helper, -cytotoxic and -memory cells.

MHC molecules first attracted interest among two groups within the scientific community. First, were those interested in studying the transplantation of tissues among unrelated individuals of the same species. These are termed allogenic transplants. They discovered a group of proteins that acted as strong antigens for the immune system. The other group of scientists were microbiologists studying restriction. They discovered that cells must see an antigen in the same "context" to recognize a previously seen
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immunosuppressed. CMV is known to be a persistent virus with direct and indirect effects on immune function. It frequently survives in a persistent state in the host, becoming clinical evident after immune suppression occurs. Groups at risk groups for CMV infection include patients undergoing cancer chemotherapy, transplant recipients, patients being treated for autoimmune disease and transplant patients.

This dissertation will look at the relationship between CMV infection and the regulation of HLA-I surface expression. The relevant literature reviewed will include that dealing with HLA-I function, structure and regulation. The biology of CMV will be reviewed. In addition, literature dealing with other known viruses which interact with HLA-I will be discussed. Lastly, research which has looked at the effects of CMV on HLA-I will be reviewed.

The working hypothesis and objectives of this dissertation will then be presented along with its clinical and scientific significance. This is followed by the methods and results presented together. The specific hypothesis and objectives are repeated in each results section. This is done for clarity since throughout this work the hypothesis evolves in terms of the previous data. Methods will stress experimental design without giving details of the techniques. This is given in Appendices in the back of the dissertation.
The discussion will review the data in terms of the original hypothesis and those developed throughout the dissertation. In addition, conflicts and agreements with other research will be examined and explained. Relevance will be discussed in terms of both clinical and scientific importance.
Human Leukocyte Antigen I

Function

HLA-I molecules are a family of proteoglycans that are associated with the cell membrane of all nucleated cells. The HLA-I molecules play an important role in cell mediated immunity. These molecules are primarily responsible for antigen presentation to cytotoxic T lymphocytes that are important for viral immunity as well as immune surveillance for neoplastic processes. HLA-I is also an important antigen in clinical rejection of transplanted tissues.

Antigen Presentation

The role of HLA-I is now understood to be as a recognition site for the association of cells from the immune system with other cells in the host. The majority of cytotoxic T lymphocytes (CTL) recognize epitopes of viral or other foreign proteins in association with HLA-I molecules.

The T-cell receptor is a polymorphic protein that has a unique binding region for each T-cell clone. This unique region gives each T-cell clone its specificity for a unique
antigen. The T-cell receptor also has a less variable region that binds to HLA-I. The HLA-I molecule provides a corresponding region that binds the T-cell receptor and a groove that holds a peptide fragment for presentation. In this way a common context is present on all cells on which antigens can be presented to the T-cell receptor.

If the T-cell receptor is able to bind the HLA-I/peptide combination tightly, an immune response is initiated. In the case of a CTL this would consist of activation of the T-cell and destruction of the cell. If no T-cell is able to bind tightly to the HLA-I/antigen complex, no immune response is created. In this way, immune reactions are not generated to self proteins.

Viral Immunity

The cardinal host response to eliminate a virus infection from within cells is the generation of cytotoxic T lymphocytes. When a virus infects a cell it subterfuges the synthetic machinery of the cell to produce viral proteins. As these proteins are synthesized a few of them are broken down, bind to HLA-I and are then transported to the surface of the cell. Circulating activated CTLs bind to these antigen/HLA-I complexes. If they are recognized by the specific T-cell then the cell is destroyed. In this way infected cells are identified and cleared.
Viruses also cause many cells to release interferons. This has an indirect effect on HLA-I because both α and β interferons increase HLA-I on most cells. In this way infected cells are better able to present viral antigens to the immune system.

**Tumor Surveillance**

Tumor surveillance is a concept which has been developed to explain the ability of CTL and natural killer cells to destroy cells which have undergone malignant transformation. It is believed that transformed cells produce proteins not normally found in cells. Since these proteins are produced within the cell, they are presented on the surface of the cell bound to HLA-I. In this way, transformed cells are identified by the immune system and destroyed.

Evidence for this theory is found in the fact that many tumors survive if they downregulate their surface HLA-I. If they are forced to express HLA-I by transfection of a non-regulated gene or by treatment with interferons, the immune system is able to destroy the cells. This remains controversial area, but most would agree that HLA-I plays some role in the progression of malignancy.

**Graft Rejection**

Autogenous and allogenic grafting has become an integral part of modern medical practice. Autogenous skin grafts were first used to provide coverage for avulsive
injuries. Soon after allogenic grafts were attempted but this was found to predictably fail and thus lead to the classic descriptions of graft rejection.

Graft rejection has been studied in many systems with human and murine being the most important. In man, the system that has been studied most extensively is the renal allograft. Rejection can occur at different time points after transplantation with different histological pictures. It has been divided into four different categories. These are hyperacute, acute, and chronic rejection.

Graft rejection can be summarized as a cell-mediated or antibody-mediated immune reaction against antigens on the transplanted endothelium. Vascular damage can be found in all rejected kidneys. This is logical since the endothelial cell is located at a pivotal location between components of the circulating immune system and the allograft. The target antigen can be HLA I, HLA II and other undefined molecules.

Allograft rejection is associated with the upregulation of MHC antigens on the allograft cells. The importance of antigens on the surface of endothelium is supported by a paper by Cerilli et al. They describe a Vascular Endothelial Cell antigen system. This is in addition to the HLA antigen system. They demonstrated vascular antibodies in related donor renal allografts which were identical at HLA-A,B,C,D and DR. In HLA-Mismatched grafts, the HLA molecules
on the vascular endothelium are the primary target. Therefore, regulation of HLA on endothelium is an important factor in the survival of transplanted tissues.

**HLA-I Structure and Biosynthesis**

**Structure**

HLA-I is present as a transmembrane heterodimer, non-covalently associated with a beta chain, termed $\beta_2$-microglobulin (MW 12,000). The gene for $\beta_2$-microglobulin is not coded for within the HLA on chromosome 6 but is found on chromosome 15.

![Diagram of HLA-I/\(\beta_2\)-microglobulin complex.](image)

**Figure 1** - Diagram of HLA-I/\(\beta_2\)-microglobulin complex.
As stated earlier, there is a high degree of polymorphism among the three loci which comprise the HLA-I genes which encodes the HLA-I proteins. The nature and purpose of this diversity is discussed later. These proteins all have a molecular weight of approximately 44,000. The primary structure consists of approximately 300 amino acids with a highly conserved sequence within and between vertebrate species. The observed diversity occurs in a specific area of the molecule which corresponds to a functional domain. There are five structural domains of a HLA-I molecule (figure 1). Domains 1, 2, and 3 are located extracellularly and each contain approximately 90 amino acids. Domains 1 and 2 are highly diverse whereas domain 3 is highly conserved. Domain 4 is the transmembrane region which contains hydrophobic amino acids flanked by hydrophilic amino acids. This domain, as well as domain 5, is highly conserved. Domain 5 is intracellular. This contains cysteine and serine residues which act as phosphorylation sites.

The structure of β2-microglobulin is strikingly similar to domain 3 of HLA-I molecules. It is non-covalently bound to domains 1 and 3. It has no intra- or trans-membrane portion. HLA-I can not exist within the plasma membrane without being associated with both β2-microglobulin and an extrinsic peptide. With few exceptions, HLA-I heavy chains
seem to need β2-microglobulin and a tightly bound peptide for their conformational integrity.\textsuperscript{12,13}

The region between Domains 1 and 2 creates a cleft which is where the peptide is bound for presentation to T-cells. This region is highly diverse. This is also the region where the T-cell receptor binds.

**Biosynthetic Pathway**

HLA-I is translated in the cytoplasm of the cell from the processed mRNA transcript. It is believed that HLA-I molecules follow the constitutive secretory pathway. They are synthesized directly into the endoplasmic reticulum, passing through the Golgi and then onto the cell surface.\textsuperscript{14} The HLA-I heavy chain folds, associates with β2-microglobulin and binds its peptide fragment prior to reaching the Golgi.

HLA-I molecules are sorted from the HLA-II molecules in the trans-Golgi reticulum (TGR). This is located between the Golgi and the cell membrane. The compartments containing the HLA-I molecules are then transported to the cell membrane whereas the vesicles containing HLA-II are fused with endosomes.\textsuperscript{15}

**HLA-I Expression and Regulation**

As stated earlier, all nucleated cells express HLA-I on their surface. However, the level of basal expression varies greatly among cell types. As a general rule, cells
within the immune system have higher densities of HLA-I on their surface than cells which are not active in immune functions. Also, areas of active inflammation have higher levels of surface HLA-I than those areas without inflammation. Although most cells do express HLA-I on their surface some cells do not. Notably, human renal tubule cells have almost no HLA-I on their surface. In contrast, normal kidney peritubular and glomerular capillaries have intense expression of HLA-I.

Many cytokines have been shown to effect HLA-I expression. Gamma interferon (γ-IFN) is the prototypical cytokine for both upregulating HLA-I and inducing HLA-II. This was first shown by Lindahl et. al. using interferons on murine leukemia cells. Other cell types have since been shown to respond to interferons including endothelial cells. Endothelial cells are important to this discussion because of their central role in graft rejection as discussed above. HLA-I increases on endothelial cells over a period of 48 hours. Endothelial cells also upregulate HLA-I in response to α-IFN and β-IFN. Interestingly endothelial cells also produce these same cytokines in response to viral infection. In this way they can act as both cytokines and as autokines.

Both tumor necrosis factor (TNF) and lymphotoxin (LT) have been shown to upregulate HLA-I expression but not HLA-II.
HLA-I Molecular Biology

Gene Structure

HLA-I molecules are coded for by three loci termed HLA-A, HLA-B, and HLA-C. These loci are closely linked on the short arm of chromosome 6. In addition they are linked to genes for the MHC HLA-II which are involved in antigen presentation and MHC class-III which are active components of the complement system. Also found among the class I, II, and III genes are the genes for the steroid 21-hydroxylase and a gene associated with hemochromatosis. The chromosomal structure is seen in figure 2.

![Chromosome 6](image.png)

**Figure 2** - Chromosome map of HLA.

The molecular structure of individual genes follows the general pattern of 8 exons with 7 intervening introns. The exon structure is closely associated with the fore mentioned domains found in the protein structure. The following
Diagram shows the known arrangement and size of introns and exons (See figure 3).

![HLA Gene Structure Diagram]

**Figure 3** - Gene map of HLA-I

Exon 1 contains the 5' untranslated sequence plus a coding sequence of 24 amino acids which codes for the hydrophobic leader sequence which is responsible for the insertion into the plasma membrane. Exon 2, 3, and 4 code for the domains 1, 2, and 3 respectively. Exon 5 codes for the transmembrane region whereas exon 6, 7, and 8 code for the cytoplasmic region. The stop codon is located either in exon 7 or 8 with the remainder of the sequence not being translated. Upstream regulating elements include the classic CCAAT sequence (CAT box) located at -20. However, none of the HLA-I genes uses the TAT box which is often seen in other eukaryotic genes. Instead of a TAT box, the human HLA-I genes have a TCTAAA sequence located at this position.
CMV - Natural History

General

CMV is a member of the herpesvirus group. It is a large double-stranded DNA virus which can cause a variety of clinical conditions. Latent CMV infection is highly prevalent in the general population. Up to 60% of healthy individuals excrete virus early in infancy.\(^2\) The virus is capable of reactivation under conditions of immunosuppression.\(^2\) As stated earlier, CMV is a clinically significant pathogen in transplant recipients. CMV mortality is 2% in renal allograft recipients and as high as 40% in bone marrow transplant recipients.\(^3\)

Host Cells

CMV has been shown to infect and replicate in many cell types, both in vivo and in vitro. CMV has been isolated from granulocytes,\(^3\) T lymphocytes,\(^3\) and monocytes which were obtained as clinical samples in patients with active CMV infection. Although CMV can be frequently isolated from polymorphonuclear cells of acutely infected patients,\(^3\) it is not clear whether this is due to replication of virus in these cells or is rather a result of nonspecific uptake of the virus. There is no change in granulocyte function in CMV-infected patients, so the presence of these virus
particles is probably not significant to these cells.\textsuperscript{35} Replication of virus has been accomplished \textit{in vitro} in T-cells particularly when these cells were engaged in a mixed lymphocyte reaction or stimulated with Interleukin 2 (IL-2).\textsuperscript{36} The same experiments failed to show replication of virus in granulocyte cell lines. The above results, then, would support the concept of T-cells as a potential site of CMV infection and replication. It is unlikely that granulocytes and macrophages are sites of infection but represent phagocytosis as part of a non-specific immune reaction.

Fibroblasts are a susceptible host to CMV \textit{in vitro} but have not been clearly demonstrated as a host cell \textit{in vivo}. Many assays for CMV rely on the ease with which CMV infects and spreads on a fibroblast monolayers. For this reason, fibroblasts have become the model system for studying CMV.

Endothelial cells have been observed to be infected with CMV in both the lungs and in the lumen of large vessels. Endothelial cells are difficult to infect with CMV \textit{in vitro} but was first reported with human umbilical vein endothelial cells (HUVEC's).\textsuperscript{37} These endothelial cells will support an infection but require high titers of CMV and produce infections which develop slowly and do not produce the classic cytopathology associated with CMV infection as in other cell types. More recently, cultured human brain capillary endothelial cells (HBCECs) were infected with CMV
strain AD169 at M.O.I of 3-5. Unlike HUVECs, HBCECs were almost as susceptible as fibroblasts to CMV infection. In addition, the expected cytopathologic lesions were observed.

Waldman has developed a strain of CMV which is more virulent for HUVECs and also demonstrates the cytopathic lesions. This CMV strain was developed by isolating clinical CMV samples and raising these viruses in endothelial cells for several generations. In this way it is felt that the natural virulence of the CMV is maintained for endothelial cells. However, this does not explain why AD169 remains virulent for HBCEC's.

Smooth muscle from the wall of arteries has been shown to act as a host for human CMV. This has been demonstrated in vivo and in vitro. Vascular smooth muscle cells seem to be a more permissive host than endothelial cells. These cells also show classic cytopathic changes in vitro. Infection of vaxular smooth muscle may be important in atheroscerotic disease and delayed rejection of cardiac transplants.

Receptors

Many viruses have been shown to infect cells by adhering to, and internalizing by way of a specific receptor. A specific receptor for CMV is postulated, since CMV binds to cells in a dose-dependent, high affinity, and saturable manner to a surface molecule. Investigators
have suggested that $\beta_2$-microglobulin is the CMV receptor. This is supported by the fact that CMV firmly binds to $\beta_2$-microglobulin. It is felt that $\beta_2$-microglobulin binds both to the CMV viral caspid and to the HLA-I of individual cells. Since $\beta_2$-microglobulin circulates freely in the plasma, it can act as a bridging protein between the virus and the HLA-I protein on the cells. Once the virus is attached to the cell, it can be internalized and infect the cell.

Some investigators feel that the binding of $\beta_2$-microglobulin by CMV may be a mechanism for free virus to evade immune detection rather than as a receptor. By coating its surface with circulating $\beta_2$-microglobulin, antigens are not exposed to circulating immunoglobulins. Neither role for $\beta_2$-microglobulin has been proven in vivo. The biological significance of these findings has not been shown.

Other specific receptors have been postulated for CMV. One laboratory has identified a protein which has a molecular weight of 92.5 kD, and another laboratory has identified a 30 kD protein as a potential receptor. The 92.5 kD peptide has been shown to bind one protein of the CMV capsid, whereas the 30 kD protein has been shown to bind the intact virion. The evidence for the 30 kD receptor includes the binding of intact CMV particles to membrane preparations from human fibroblasts, Raji cells and Daudi
cells, and showing that the responsible protein is a 30 kD protein. CMV was also shown to bind to western blots to with a 30 kD protein. However, they were also able to demonstrate binding to the 92.5 kD protein. Finally, they purified the 30 kD protein through denaturing methods, reassembled it and then were able to demonstrate binding to the intact virion. At this time the 30 kD glycoprotein is the most likely receptor for CMV.

**Molecular Biology**

CMV transcription can be divided into three separate phases: immediate early (IE), early (E), and late (L). The major gene product of the IE genes is a 64kD protein which has been cloned and sequenced. It is hypothesized that this protein acts as a regulatory protein for later viral gene expression, and it has been shown to be a trans-activator of genes in the early region. It differentially activates three promoters for the early genes. In this way the IE genes act as the regulatory elements for the Early genes.

The IE genes are transcribed from the major IE promoter. They are under control by factors which influence host cell transcription. These factors are probably related to cell replication since it has been shown that cell division is required for the replication of CMV. IE genes are transcribed in the absence of viral protein
synthesis and therefore rely only on the host's transcription machinery. During latency, only genes in the IE regions are transcribed and translated. CMV-specific CTLs are able to recognize products of the IE region. This is important since this would allow the immune system to identify cells which have been recently infected.

CMV in Transplantation

Patients undergoing long-term immunosuppression after organ transplantation frequently suffer reactivation of latent or persistent viral infections. The most important viruses in the immunosuppressed population are in the Herpes family, and of these CMV is the most important pathogen. It has been shown that CMV infections affects nearly two-thirds of all transplant recipients. It is thought that CMV, in and of itself, is capable of decreasing immune competence. This is seen through severe leukopenia, impaired cell-mediated immunity (as defined by responsiveness to skin test antigens or by lymphocyte responses in vitro), changes in circulating T-cell subsets and impaired alveolar macrophage function.

It has long been felt that CMV infection has direct toxic effects on transplanted organs. However, a direct link between CMV and transplant rejection has not been
demonstrated. There are several studies which have shown that those patients which have a CMV infection after grafting have decreased survival of their allograft.\textsuperscript{64, 65, 66} Patients suffering an episode of CMV infection after their transplant have increased chances of acute and chronic graft rejection.

More direct evidence for CMV allograft toxicity exists. CMV is thought to be associated with a lesion in the glomeruli of transplanted kidneys.\textsuperscript{67} This lesion correlates well with episodes of CMV infection but it has not been proven that CMV is responsible for this lesion or that CMV particles exist within this lesion.\textsuperscript{68, 69} Antigens have been found in the allografts of individuals suffering from a CMV infection\textsuperscript{70} as well as circulating in their peripheral blood.\textsuperscript{71} More recently, CMV has been found in allograft renal biopsies in patients suffering from an active CMV infection.\textsuperscript{72}

An indirect effect of CMV infection is the release of $\alpha$- and $\beta$-interferons. As discussed before, both of these cytokines lead to upregulation of HLA-I on the surface of cells. This could then lead to an increased number of antigenic targets for alloreactive T-cells. In humans, renal transplant recipients suffering from CMV infection have been shown to have increased HLA-II in their allografts. This has been associated with episodes of graft rejection.\textsuperscript{73} Both upregulation of HLA-I and HLA-II in grafted organs
could be due to an indirect effect of CMV infection. This could be through the production of interferon by infected cells. Interferons lead to upregulation of HLA-I and HLA-II which could then provide a mechanism leading to graft rejection.

Another mechanism for increased graft rejection could be cross-reactivity between viral proteins with alloantigens. This would provide a more direct mechanism for injury of the allograft during CMV infection. It has been demonstrated that antibodies can be raised to both the HLA-DR beta chain and to a glycoprotein coded for by an immediate early gene of CMV.74 There is also a CMV viral protein which has been shown to be homologous to HLA-I.75 T-cells which react to a CMV infection could then cross-react with the allogenic HLA-I, leading to graft rejection. This could explain the lack of CMV found in kidneys undergoing rejection. T-cells could be activated at a distant site and then react to the alloantigens in the kidney.

There is some evidence to show that CMV infection has no effect on the outcome of renal allografts. This principally exists in a rat model in which it was shown that CMV infection in intact and immunosuppressed rats had no effect on the survival of the allograft or of the rat.76
HLA-I Viral Interactions

The classic example of viral/class I interactions is the presentation of viral antigens in association with the class I glycoprotein on the cell's surface. The presence of these antigens, along with the recognition region of the HLA-I molecule, allows T-helper cells and T-cytotoxic cells to initiate and carry out an immune response. It would seem possible that some viral species would evolve mechanisms to evade detection by the immune system. This would include mechanisms to alter the surface expression of HLA-I. It is also possible that cells would develop mechanisms by which they could upregulate their surface expression of HLA-I to better present viral antigens once they have become infected.

Adenovirus type II

The adenovirus type II was the first virus which was shown to interact with the HLA-I protein in a way to promote the viral infection. The adenoviruses are a group of DNA viruses which cause upper respiratory infections in humans and some other species. This virus' genome codes for approximately 50 proteins. The expression of these genes is divided into an early and late phase which is demarcated by the onset of viral DNA synthesis. One of the proteins coded for in the early region is termed E3/19K glycoprotein.
It has been shown that this protein forms a complex with the HLA-I protein. By transfecting cells with the gene for E3/19K, it has been demonstrated that the presence of this glycoprotein prevents surface expression of HLA-I on the plasma membrane. The presence of HLA-I in the cytoplasm was demonstrated by immunoprecipitation, and the absence of HLA-I on the surface was demonstrated by flow cytometry. This lack of expression of HLA-I has been shown to be functionally significant, since it leads to lack of lysis by AD-2 primed lymphocytes. HLA-I can be restored in infected cells by treatment with γ-IFN. Lysis by primed lymphocytes can then be restored by upregulating HLA-I through treatment of infected cells by γ-IFN.

**Epstein-Barr Virus**

Epstein-Barr virus (EBV) also has been shown to effect the expression of HLA-I on the cell's surface. It has been shown that cells infected with EBV are resistant to lysis by HLA-I antigen-restricted CTL's. This resistance was found to parallel down regulation of HLA-I in various cell lines. It is felt that this downregulation allows EBV-transformed cells to escape immunologic surveillance.

**Herpes Simplex Virus**

Herpes Simplex Virus (HSV) has also been shown to interact with HLA-I. It was observed that cells infected
with HSV type 2 (HSV-2) are less susceptible to lysis by CTL's than are cells infected with HSV type 1 (HSV-1) even though both viruses produce similar antigens on the surface of cells, and these antigens are cross-reactive for the T-cells used. This resistant property of HSV-2 cells has been mapped to a position on the viral genome but the nature of this loci is unknown. Experiments have been performed to measure the presence of HLA-I on the surface of HSV-infected cells. It was discovered that both HSV-1- and HSV-2-infected cells had decreased surface expression of HLA-I but that HSV-2 had a far greater decrease. Furthermore, by looking at recombinants of HSV-1 and HSV-2 it was discovered that this property of decreased surface HLA-I mapped to the same location as the resistance to CTLs. This provides good evidence that HSV-2 has developed a mechanism by which it can decrease surface expression of HLA-I and thereby avoid detection by the immune system.

Lymphocytic Choriomeningitis Virus

Viral persistence in neurons has been looked at in a mouse model using lymphocytic choriomeningitis virus (LCMV). It was observed that specific CTL's to LCMV were unable to lyse infected neurons in the brains of mice despite the presence of viral antigens on their surface. However, cells of the leptomeninges which were infected were quickly destroyed.
An *in vitro* system was used to study the mechanism by which the neurons were able to escape destruction by CTLs. Infected fibroblasts and OBL21 cells were used in these experiments as a control. OBL21 is a cell line with neuronal characteristics including the expression of neurofilaments, neuron-specific enolase and voltage-dependent potassium channels. Like the *in vivo* system, both of these cells could be infected with LCMV, but only the fibroblasts could be lysed by specific T-cells. OBL21 cells were shown to not express HLA-I on their surface, whether or not they were infected with virus. However, if they were treated with γ-interferon, they expressed HLA-I on their surface and could then be lysed by specific CTLs. Surface HLA-I was also increased by transfecting the cells with the gene for HLA-I and again this restored the sensitivity to lysis by CTLs. This is an example, then, of a virus which persists in neurons due to their lack of normal HLA-I expression. This virus is unable to regulate the OBL21 cells HLA-I but takes advantage of the decreased HLA-I in neurons to evade detection and persist.

**CMV**

The effect of CMV on HLA-I expression has been examined by other investigators. One group has looked specifically at the effect of the class I homologue encoded for with the CMV genome on HLA-I expression in the hosts cell. They
showed that transfected class I homologue would bind to transfected β2-microglobulin and would be transported to the cell's surface. Neither the β2-microglobulin or the HLA-I homologue was expressed on the surface if either was transfected alone. This would indicate that the class I homologue is functional in that it can bind the appropriate molecule and can then be expressed on the cell's surface. It is unknown whether this complex is functional in antigen presentation. The authors do hypothesize that this HLA-I homologue could go on to coat viruses, taking β2-microglobulin with it, which could then use HLA-I on uninfected cells as a receptor.

Several authors have looked at the effect of CMV infection on both HLA-I and HLA-II in different model systems. The results of these experiments have produced mixed results. Grundy et al.90 demonstrated an increase in HLA-I in fibroblasts whereas van Dorp et al.91 demonstrated an increase in HLA-I in endothelial cells.

Tudor also looked at expression of HLA-I on endothelial cells.92 They used a functional assay by measuring the effect on the binding of peripheral blood mononuclear cells to the infected cells. In their model system they infected HUVECs with AD 169. The infection was allowed to progress for 1 week. At the end of that time, the level of infection was evaluated by immunohistochemistry and was found to be less than 5%. Expression of HLA-I and
B2-microglobulin was assayed by Enzyme-Linked Immunosorbent Assay (ELISA). Their results showed a 25% increase in HLA-I expression with no increase in Factor VIII. They also showed that there was a 30% increase of PBMC binding after two hours of co-incubation. They attributed this increase in PBMC binding to the increase in HLA-I expression.
HYPOTHESIS

The literature reviewed shows that HLA-I expression is important for control of viral infection and the lack of HLA-I surface expression is apparently important for viral persistence. The literature also documents that CMV is a known persistent virus. In addition HLA-I is important in transplantation. The degree of HLA-I mismatch is probably the leading predictor of graft rejection. CMV has also been associated with graft rejection. This had lead authors to speculate on HLA-I upregulation as being a mechanism for graft rejection.

The literature on the effects of CMV infection surface HLA-I expression are mixed and confusing. This is probably due to model systems which obtain low levels of infection. This had made research on mechanisms of upregulation and downregulation even more unclear.

The purpose of this dissertation will be to examine the direct and indirect effects of CMV on viral infection and then look at possible mechanism for these changes.
METHODS/RESULTS

Surface HLA-I Expression

Hypothesis

The hypothesis based on the literature reviewed above is that CMV-infected cells upregulate their surface HLA-I.

Methods

Two in vitro model systems were used to test this hypothesis. Fibroblasts are the cell type used to culture clinical CMV, and they are the prototypical cell type in which to study CMV in the laboratory. Therefore, we used fibroblasts to enable comparison between our work and that performed in other laboratories. Infections can be produced quickly in fibroblasts, with a large proportion of the cells being infected in a short amount of time. This enables large-scale, reproducible experiments to be performed.

Fibroblasts used in these experiments were MRC-5. MRC-5 is a human fibroblast cell line available through American Type Culture Collection. This cell line was derived from normal lung tissue of a 14-week-old male fetus. These cells were grown in culture as discussed in Appendix A.
Cells were grown in 150 cm² flasks until confluent. They were infected with the CMV strain VHL-F. VHL-F is a CMV viral strain developed by Waldman. Its characteristics are similar to other known CMV strains. It was grown from a clinical isolate and maintained in MRC-5 cells for 15 passages.

Endothelial cells were used as a more biologically and pathologically relevant cell type. Endothelial cultures become infected more slowly than fibroblasts and a persistent, chronic infection results.

The type of endothelial cells used in these experiments were human umbilical vein endothelial cells (HUVECs). These cells were isolated as primary cultures, characterized and maintained as discussed in Appendix A. VHL-E was the CMV strain used for these experiments. This virus was developed by Waldman to retain its normal virulence and biological behavior when grown in endothelial cultures. It produces classic cytopathologic lesions in endothelial cells, and a high degree of infection can be achieved.

Surface expression was assayed by flow cytometry. Live cells were reacted with the monoclonal antibody W6/32. This antibody binds to the intact HLA-I in complex with β2-microglobulin (For specific technique see Appendix B). Since the cells in these experiments were vital and not permeable to the antibody, only surface expression was measured. An isotypic control antibody, L26, was used to determine non-specific binding. Two staining methods were used, an
indirect and direct method. Both methods are described in the Appendix. These two methods gave similar results, with slightly lower background reactivity seen using the direct method.

Aliquots of cells from the same population of cells as those used for flow cytometry were used to prepare cytocentrifuge slides (See Appendix B for details of technique). These cells were then stained by immunohistochemistry with the monoclonal antibody ENP. This allowed identification and counting of the CMV infected cells to determine the percentage of infected cells for each experiment.

Results

Fibroblasts

Cultures of fibroblasts were infected with varying inocula of virus for varying periods of time. The cells were followed in situ through an inverted, phase contrast microscope. Typical cytopathic changes could be observed in the cells. The following photomicrographs show a typical culture of fibroblasts without infection (Figure 4) and a typical culture that is highly infected (Figure 5).

These photomicrographs show the classic pattern of cytoplasmic inclusions and multinucleated giant cells associated with CMV infection. These infections progressed
quickly with all the cells in a flask showing signs of infection within three days and most cells dying in a week.
**Figure 4** - Phase contrast photomicrograph of MRC-5 cells in culture. Original magnification 40x. Control culture for cells in Figure 5.

**Figure 5** - Phase contrast photomicrograph of MRC-5 cells infected for 3 days with 10,000 pfu of VHL-F. Original magnification 40x.
The cultures were harvested by trypsinization and cytocentrifuge slides prepared. These were then examined by immunohistochemistry with the monoclonal antibody ENP. This antibody is specific for Early Nuclear Protein. It gives a nuclear staining pattern in CMV-infected cells.

Figure 6 and Figure 7 showed the typical immunohistochemical staining seen in these cultures. The peroxidase reaction, which gives a reddish brown color change. The non-infected culture (figure 6) showed no specific reaction pattern. The CMV-infected culture (figure 7) shows a strong reaction in the nucleus of virtually every cell. Immunohistochemical reaction with ENP which allowed easy counting of infected and non-infected cells. This in turn allowed the reporting of the percent infection for each specific culture.

These preliminary studies demonstrate that we are able to produce a high level of infection in fibroblasts which are consistent with those described in the literature. We are also able to follow the infections in situ through phase contrast microscopy and we are able to use immunohistochemistry with a specific antibody to assay for the level of infection.
Figure 6 - Photomicrograph of a CMV infected culture of MRC-5 cells. Stained by immunohistochemistry with antibody ENP. Original magnification 40X.

Two patterns of HLA-I surface expression were observed by flow cytometry. The pattern observed depended upon the length of time after infection and the concentration of virus in the inoculum. Figure 8 shows two histograms of W6/32 binding, with and without CMV infection, in fibroblasts. These cultures were infected with a high concentration of virus (10,000 pfu) for 3 days. The ENP staining showed that the infected culture was 95% infected with CMV.

The histogram shows log intensity on the x-axis and frequency on the y-axis. W6/32 is plotted with the solid line whereas the control antibody is the dashed line.
Figure 7 - Flow cytometry histograms of HLA-I expression in non-infected MRC-5 cells on the left and highly infected MRC-5 cells on the right. X-axis is log fluorescence, Y-axis is frequency. Dotted lines are the isotypically matched control.

These results show a distinct decrease in the expression of HLA-I. This is seen as a shift to the left of the curve in the above histogram. However, some cells do remain positive as demonstrated by some cells still being to the left of the histogram. Statistical analysis of the above histograms with a Students T-test shows that the observed decrease in surface expression is statistically significant (p<0.001).

This experiment was repeated five times with the same inoculum and time after infection. Similar appearing histograms were obtained for each of these experiments. The mean fluorescence was determined for each of these experiments from the linear scale of the flow cytometer. Table 1 shows the results for each experiment with the
average and standard deviation. Figure 9 shows a bar graft of these mean fluoresences for both HLA-I and control antibodies.

These results show that a highly CMV-infected culture of fibroblasts has a definite decrease in surface expression of HLA-I proteins at three days. A Students T-test comparing the averages for these five experiments shows the difference to be significantly different (p<0.001).
Table 1 - Net mean fluorescence of MRC-5 cells infected with 10,000 pfu of CMV for three days. Reacted with W6/32 and L26 antibodies.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Net W6/32 Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CMV- 140.0</td>
</tr>
<tr>
<td>2</td>
<td>CMV+ 7.0</td>
</tr>
<tr>
<td>2</td>
<td>CMV- 147.0</td>
</tr>
<tr>
<td>2</td>
<td>CMV+ 22.7</td>
</tr>
<tr>
<td>3</td>
<td>CMV- 181.8</td>
</tr>
<tr>
<td>3</td>
<td>CMV+ 9.2</td>
</tr>
<tr>
<td>4</td>
<td>CMV- 151.3</td>
</tr>
<tr>
<td>4</td>
<td>CMV+ 47.6</td>
</tr>
<tr>
<td>5</td>
<td>CMV- 128.0</td>
</tr>
<tr>
<td>5</td>
<td>CMV+ 85.1</td>
</tr>
<tr>
<td>Average</td>
<td>CMV- 149.6 ± 20.0</td>
</tr>
<tr>
<td></td>
<td>CMV+ 34.3 ± 32.3</td>
</tr>
</tbody>
</table>

Figure 8 - Net mean fluorescence of W6/32 binding to non-infected and CMV-infected MRC-5 fibroblasts. Cells were infected for 3 days with 10,000 pfu of virus. N=5.
The next set of experiments looked at lower concentrations of viral inoculum and earlier time points. This was to ascertain whether there was a dose-response phenomenon and to study the kinetics of the decrease in class I.

Ten cultures were divided into three treatment groups, and one culture served as a control. Each treatment group received a different inoculum of virus: 10,000 pfu, 1000 pfu or 100 pfu per ml. Each treatment group was further divided into three time points of 24, 48, and 72 hours. Flow cytometry was performed on cells from each culture, and cytocentrifuge slides were made to determine the level of CMV infection. These experiments were performed in triplicate.

The following table (Table 2) and line graph (Figure 10) show the net mean fluorescence for each of the treatment groups for each of the time points. The net mean fluorescence was determined by subtracting the control antibody mean fluorescence from the HLA-I antibody mean fluorescence. The mean fluorescence was determined from the linear scale of the flow cytometer.
Table 2 - Net mean fluorescence of MRC-5 cells reacted with HLA-I antibody W6/32. Cells are infected with the indicated concentration of virus for the indicated length of time. Results are an average of three experiments.

<table>
<thead>
<tr>
<th></th>
<th>0 Hours</th>
<th>24 Hours</th>
<th>48 Hours</th>
<th>72 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 PFU</td>
<td>152.3 ± 22.4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>10,000 PFU</td>
<td>N.D.</td>
<td>96.1 ± 13.2</td>
<td>35.0 ± 11.3</td>
<td>13.0 ± 8.5</td>
</tr>
<tr>
<td>1,000 PFU</td>
<td>N.D.</td>
<td>97.8 ± 4.3</td>
<td>92.4 ± 47.4</td>
<td>63.0 ± 12.4</td>
</tr>
<tr>
<td>100 PFU</td>
<td>N.D.</td>
<td>122.7 ± 28.4</td>
<td>119.8 ± 15.9</td>
<td>51.3 ± 34.1</td>
</tr>
</tbody>
</table>

Figure 9 - Net mean fluorescence of MRC-5 cells reacted with HLA-I antibody W6/32. Cells are infected with the indicated concentration of virus for the indicated length of time. Results are an average of three experiments.
The highest concentration of virus (10,000 pfu) showed a steady decrease in surface HLA-I over the 72 hours of the experiment. Both the lower concentrations, 1000 pfu and 100 pfu, showed decrease in surface HLA-I during the first 24 hours but then showed a leveling off between 24 and 48 hours with a decrease resuming at the 72 hours. The mean fluorescence at 72 hours was always significantly less than control for all concentrations of virus (p<0.001)

Figure 11 shows two histograms from these experiments. The left histogram is the non-infected control and the right histogram is a culture which was infected for 48 hours with 1000 pfu of CMV virus. The vertical line is arbitrarily placed to mark the peak of the control curve. The line was then placed at the same horizontal position on the infected culture's histogram. This illustrates an important point. It is easy to see that there are two populations in the infected flask. There is a large peak of more positive cells to the right and a smaller population of more negative cells to the left. The overall mean fluorescence for the two cultures was 209.8 for the non-infected culture and 193.7 for the infected culture. This would indicate that the average did not change significantly, but it would appear that there are two populations which have both responded to the infection in opposite ways. These dual peaks were a consistent finding in flasks infected with 1000 pfu of virus for 24 and 48 hours. They were never seen at 10,000 pfu of virus.
Figure 10 – Flow cytometry histograms of MRC-5 cells. The left histogram is a non-infected control culture and the right is infected with 1000 pfu of CMV for 48 hours.

These results demonstrate that moderate to low inocula of CMV, at 24 to 48 hours, cause a biphasic effect on the expression of surface HLA-I in fibroblasts cultures. There is a population which upregulates its surface HLA-I and a population which downregulates its surface HLA-I.

The following two histograms demonstrate the extent that HLA-I upregulation can be seen. Figure 12 shows a control culture on the left and a culture infected with 100 pfu of CMV for 24 hours on the right. At this early time point, with a low level of infection, there is virtually no downregulation seen, but considerable upregulation. The upregulation is seen as the shoulder extending to the right of the main peak. This was only seen in cultures with this very low inoculum. In this particular culture only 1% of the cells were infected.
Figure 11 - Flow cytometry histograms of MRC-5 cells. The left histogram is a non-infected control culture and the right is infected with 100 pfu of CMV for 48 hours.

Since this culture had such a low level of infection it is unlikely to be a direct effect of CMV infection. The opposite is true of a flask which is almost 100% infected. These cultures show almost universal downregulation of their surface HLA-I. Therefore, the hypothesis which is supported by this data is that CMV infection of individual cells leads to downregulation of that cell's surface HLA-I. Furthermore, CMV infection of a cell leads to upregulation of other cells in the same culture which are not infected. This is most likely due to the release of interferons by the infected cells which leads to upregulation of HLA-I. The release of interferons by infected cells and the upregulation of HLA-I by interferons are a known phenomenon and was not further investigated.
Endothelial Cells

Surface expression of HLA-I was also studied in endothelial cells. This is a more relevant cell type both in terms of the \textit{in vitro} behavior of the virus as well as being more important for pathological processes. As in the fibroblasts, flow cytometry was used to assay for surface expression HLA-I protein, and immunohistochemistry was used to assay for the level of CMV infection.

Pairs of cultures were set up by dividing an established culture of HUVEC's into identical cultures. One flask was inoculated with VHL-E and the other flasks acted as the control. Cultures were observed through an inverted phase-contrast microscope to monitor the level of infection in the cultures.

Figure 13 and 14 show photomicrographs of HUVECs in culture. Figure 13 is a non-infected culture whereas Figure 14 is highly infected with CMV. As in the fibroblasts, the typical cytopathic changes associated with CMV infection are seen. This includes viral inclusions and multinucleated giant cells. These infections progressed much slower than the infections seen in the fibroblasts. This particular photograph is of a culture 10 days post-infection. These infections progressed at a variable rate. This made timed experiments impractical but enabled the culture to be studied in a relative steady state for various levels of CMV infection. These infections typically took two weeks to
involve all the cells in the flask, and a large proportion of cells would live for as long as three weeks.

Infection of HUVEC's could also be demonstrated by immunohistochemical reaction using the antibody ENP. This is shown in the photomicrographs in Figure 15 and Figure 16 which shows non-infected and CMV-infected HUVECs. As in the fibroblasts, these cells show the classic reaction pattern with nuclear staining. These cells could be easily counted and the percent infection calculated.
Figure 12 - Phase contrast photomicrograph of a HUVECs in culture. Original magnification 40X. Control culture for cells in figure 13.

Figure 13 - Phase contrast photomicrograph of a HUVECs highly infected with VHL-E. Original magnification 40X.
Figure 14 - Photomicrograph of CMV-infected HUVEC culture. Reacted with antibody ENP. Original magnification 40X. Brownish-red staining indicates early nuclear protein.

Flow cytometry of all HUVEC cultures infected with CMV revealed decreases in surface expression of HLA-I. This demonstrated in Figure 17 which shows a moderately infected culture.
Figure 15 - Flow cytometry of non-infected HUVECs on the left and CMV-infected HUVECs on the right. Antibody is W6/32 with L26 as the nonspecific control.

These histograms show a shift to the left, representing a decrease in the surface expression of HLA-I. This was found to be statistically significant using a students T-test to compare the means (P<0.001).

This experiment was repeated six times with different levels of resulting infection. This data is shown in Table 3. The net mean fluorescence was calculated by subtracting the mean fluorescence of the control antibody from W6/32. The percent decrease in fluorescence was then calculated by dividing the difference between the non-infected and CMV-infected net mean fluorescence by the non-infected net mean.
Table 3 - Results of flow cytometry for pairs of HUVEC cultures with and without CMV infection. Stained with mAb W6/32 and L/26. Data is net mean fluorescence taken from the linear scale.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Cont.</th>
<th>CMV +</th>
<th>% Dec.</th>
<th>%CMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>112.3</td>
<td>82.8</td>
<td>60.8</td>
<td>26</td>
</tr>
<tr>
<td>B</td>
<td>65.9</td>
<td>15.4</td>
<td>40.3</td>
<td>77</td>
</tr>
<tr>
<td>C</td>
<td>54.2</td>
<td>10.7</td>
<td>45.2</td>
<td>80</td>
</tr>
<tr>
<td>D</td>
<td>123.9</td>
<td>82.8</td>
<td>2.1</td>
<td>33</td>
</tr>
<tr>
<td>E</td>
<td>194.6</td>
<td>52.1</td>
<td>68.7</td>
<td>73</td>
</tr>
<tr>
<td>F</td>
<td>172.5</td>
<td>4.8</td>
<td>70.3</td>
<td>97</td>
</tr>
</tbody>
</table>

Figure 16 - Scatter diagram of percent infection on the X-axis and percent decrease in HLA-I on the Y-axis. Data taken from Table 3.
Figure 18 shows the result of plotting the percent infection on the X-axis against the decrease in HLA-I expression on the Y-axis. This gave a positive correlation with a Pearson coefficient of determination of 0.819.

**Conclusion**

This data shows a relationship between CMV infection and a decrease in the surface expression of HLA-I. This was observed both in fibroblasts and in endothelial cells. In fibroblasts, two populations of cells were observed when low and moderate inoculums of CMV were used. At high inocula only decreases in HLA-I were observed. Endothelial cells always showed decreases in surface HLA-I which were proportional to the level of infection.

These results taken together force rejection of the hypothesis that CMV infection up-regulates surface HLA-I. The only up-regulation seen was at low levels of infection. This is consistent with a new hypothesis that CMV infection leads to down-regulation of HLA-I with the release of interferons. The release of interferons leads to up-regulation in non-infected cells in the same culture.
Cytoplasmic HLA-I Expression

Hypothesis

There are two general explanations for the decrease in HLA-I on the surface of the cells. The first is that the protein is present in the cells but is not on the surface. Infection with CMV could lead to internalization of HLA-I from the surface or prevent the protein from being transported to the surface. Both of these mechanisms would give normal to increased cytoplasmic levels of HLA-I.

The other explanation is that HLA-I protein is not present in the cell at all. This could be due to a decrease in HLA-I mRNA, a failure to translate HLA-I mRNA, or decreased half-life of the protein. These would all lead to decreased cytoplasmic levels of HLA-I. Therefore, the following experiments were designed to look at cytoplasmic expression of HLA-I to test these possible hypotheses.

Methods

Immunohistochemistry of acetone-fixed cells allows the visualization of molecules on the surface and in the cytoplasm. The following experiments assayed both fibroblasts and endothelial cells stained by these methods. As in the above experiments, ENP was used to identify the percent of infected each experiment. W6/32 was used to test for HLA-I. Dual staining was performed to identify the
phenotype of individual cells in terms of CMV−/CMV+ and HLA-I+/HLA-I−.

HLA-I was quantitated by examining 100 cells on each slide. Each cell was assigned a number from 0-3 on the following scale:
0  - No HLA-I reaction
1  - Decreased HLA-I reaction
2  - Normal HLA-I reaction
3  - Increased HLA-I reaction

Normal staining was defined as the average staining observed on the noninfected HLA-I stained slide. Data is displayed in frequency histograms. Statistical analysis consisted of a Mann-Whitney test to compare the infected culture to its non-infected control.

Results

Fibroblasts

Cultures of fibroblasts were infected with 10,000 pfu, 1000 pfu or 100 pfu of VHL-E CMV. The infections were then allowed to progress for one, two, or three days and the cells then harvested with trypsin/EDTA. Non-infected control cultures were harvested for each experiment.

Cytocentrifuge slides were prepared as described in Appendix C. These slides were then stained for HLA-I or W6/32.
Figure 17 shows a photomicrograph of a non-infected culture of fibroblasts. Figure 18 shows a culture infected with 10,000 pfu of VHL-F CMV for three days. The corresponding flow cytometry revealed an 85% decrease in surface HLA-I by flow cytometry. ENP immunohistochemistry revealed 94% of the cells positive for CMV infection.

The W6/32 staining shows a remarkable decrease in both surface and cytoplasmic staining in the infected cells when compared to controls. This photo shows most of the cells in the culture to be without any staining and only a few with decreased or normal staining. No cells seem to have upregulated their HLA-I. The large cytopathic cells almost always show decreased or no HLA-I staining.
Figure 17 - Photomicrograph of a non-infected culture of MRC-5 fibroblasts. Stained by immunohistochemistry with mAB W6/32. Original Magnification 40X. This was the control culture for figure 18.

Figure 18 - Photomicrograph of a culture of MRC-5 fibroblasts infected for 72 hours with 10,000 pfu of VHL-F. Stained by immunohistochemistry with mAB W6/32. Original Magnification 100X.
Cell counts were performed as described above and the results are shown in the following graph (figure 21). This shows the results of cells infected with 10,000 pfu of CMV VHL-F, from 24 through 72 hours. The results show a dramatic decrease in HLA-I as early as 24 hours. The phenomenon is almost complete by 48 hours and is not statistically different than the control antibody by 72 hours.
Figure 19 - Histogram of HLA-I staining intensity of non-infected and CMV infected fibroblasts. MRC-5 cells were infected with 10,000 pfu of VL-F for 0, 24, 48 and 72 hours.

Figure 20 - Histogram of HLA-I staining of non-infected and CMV-infected fibroblasts. MRC-5 cells were infected with 1000 pfu of VHL-F for 0, 24, 48 and 72 hours.
Figure 21 - Histogram of HLA-I immunohistochemistry of non-infected and CMV-infected fibroblasts. MRC-5 cells were infected with 100 pfu of VHL-F for 0, 24, 48 and 72 hours.

These results are consistent with the results seen by flow cytometry in fibroblasts. In those experiments both up-regulation and down-regulation were seen with 1000 to 100 pfu of CMV virus, whereas at 10,000 pfu only down-regulation was seen.

**Endothelial Cells**

HUVECs were infected with VHL-E. 7-14 days after infection the cells were harvested by trypsinization, depending on their appearance by phase contrast microscopy. Cytocentrifuge slides were prepared and reacted with W6/32 and for CMV with ENP. Cells were scored for level of HLA-I
as described for fibroblasts and scored for presence or absence of CMV.

The photomicrograph in figure 24 shows a non-infected culture of HUVECs stained by immunohistochemistry for HLA-I. Figure 25 shows a photomicrograph of fibroblasts infected with 10,000 pfu of CMV for 72 hours. This culture was shown by ENP immunohistochemistry to be 95% infected with CMV. Flow cytometry with antibody W6/32 showed an 85% decrease in surface HLA-I.

The non-infected culture has even staining with almost all the cells showing a similar staining pattern. The infected culture however, shows many cells with no staining at all and many cells with decreased staining. There are a few normal staining and a virtually no cells with increased staining. The large cytopathic cells are usually without any staining for HLA-I. These results show that both surface and cytoplasmic expression of HLA-I protein are downregulated at this high level of infection.
Figure 22 - Photomicrograph of a non-infected culture of HUVECs. Stained by immunohistochemistry with mAB W6/32. Original magnification 40X. Control culture for figure 23.

Figure 23 - Photomicrograph of a CMV-infected culture of HUVECs infected with VHL-E CMV. Stained by immunohistochemistry with mAB W6/32. Original magnification 40X.
The following graph, figure 26, shows the results of scoring immunochemistry slides as described above. The results are from a non-infected and CMV-infected culture. This culture had a 97% decrease in surface HLA-I by flow cytometry and 85% of the cells in the culture were ENP positive. Figure 27 shows a similar histogram from a different experiment. This is a culture which was shown to be 64% infected with CMV by ENP immunohistochemistry and had a 73% decrease in surface HLA-I.

The results show a decrease in HLA-I in both graphs. Both graphs do show a slight increase in the number of upregulated cells. In the first case, this increased was from 2% to 5%. In the second culture they increased from 1% to 8%. A Mann-Whitney U test shows a statistically significant (p<0.01) decrease in HLA-I for both populations.
Figure 24 - Histogram of HLA-I staining intensity of non-infected and CMV-infected HUVEC's. The infected culture had a 97% decrease in surface HLA-I expression by flow cytometry.

Figure 25 - Histogram of HLA-I staining of non-infected and CMV-infected HUVEC's. The infected culture had an 85% decrease in surface expression of HLA-I by flow cytometry.
Conclusion

These results show a definite decrease in HLA-I expression both in CMV-infected fibroblasts and endothelial cells. Again, there are a minority of cells which seem to show up-regulation. However, it is possible to reject the hypothesis that the mechanism of down-regulation is by either internalization of HLA-I protein or failure of HLA-I protein to be transported to the surface.
Correlation of CMV with HLA-I Expression

Hypothesis

Both surface expression and cytoplasmic expression of CMV-infected cultures show some cells which seem to up-regulate or retain their expression of HLA-I. Since not all of the cells are infected, and the phenomenon is more pronounced in cultures with lower levels of infection, it is hypothesized that the non-infected cells upregulate their HLA-I in response to the presence of infected cells in the same culture. The following experiments allow cells to be identified by whether they are CMV positive or negative and also whether HLA-I expression is normal, upregulated or downregulated. As in single staining, the HLA-I identifies both cytoplasmic and surface HLA-I.

Methods

Cytocentrifuge slides were prepared as described in the previous experiments. They were stained using the dual stain immunohistochemical method described in Appendix B. The immunoperoxidase reaction was used with the mAb W6/32 which is specific for HLA-I and the alkaline phosphatase reaction was used with the mAb ENP. This results in slides with reddish brown stain identifying HLA-I and blue stain which identifies CMV-infected cells.
Results

Fibroblasts

A culture of MRC-5 fibroblasts was infected for 48 hours with 1000 pfu of VHL-F CMV virus. This inoculum and time point was chosen because it was shown in previous experiments to give upregulated, downregulated and normal cells HLA-I by flow and single staining immuno-histochemistry. By dual staining this culture, it will be possible to tell whether the up-regulated cells are CMV+ or CMV- and whether the down-regulated cells are CMV+ or CMV-.

Figure 28 shows a photomicrograph of a dual-stained cells from this culture. It shows approximately half of the cells in the culture are positive for CMV. This is shown by the blue staining nuclei. This culture also shows the uneven staining for HLA-I which was previously seen in cultures with this level of infection. There are some cells with decreased HLA-I, some with increased HLA-I and some with no change in their HLA-I expression. Again, the small cells tend to be the ones with increased HLA-I, whereas the large cells tend to have decreased HLA-I. As predicted, virtually all of the cells with decreased HLA-I expression are positive for CMV and virtually all the small cells with increased HLA-I are CMV-negative.
Figure 26 - Photomicrograph of dual-stained CMV-infected MRC-5 fibroblasts. The red stain identifies HLA-I stained with the mAB W6/32 and the blue stain identifies CMV-infected cells stained with the mAB ENP.

The above culture was quantitated by scoring the amount of HLA-I staining on a scale from 1-4 as described before. In addition each cell was also scored as CMV- or CMV+. This gives eight possible outcomes for each cell scored. The results of an average of three different counts is shown in the following figure.

This graph shows that the majority of the CMV positive cells have either decreased or no HLA-I expression whereas the majority of CMV negative cells have either normal or upregulated HLA-I. This supports our hypothesis that CMV-infected cells down-regulate their HLA-I and produce
interferons which leads to up-regulation in HLA-I by non-infected cells within the same culture.

Figure 27 - Histogram of a single culture of MRC-5 fibroblasts infected with 1000 pfu of CMV for 48 hours. Dual stained for HLA-I and CMV. Each cell is categorized for CMV positivity and HLA-I staining intensity.

Endothelial Cells

A similar experiment was also performed in endothelial cells. A culture of HUVECs was infected with VHL-E and followed for eight days, at which time most of the cells in the flasks appeared to be infected. The cells were harvested and the cells used to produce cytocentrifuge slides. These slides were then stained by the dual stain immunohistochemistry technique. A photomicrograph of one of these slides is shown in Figure 31.
Figure 28 - Photomicrograph of dual stained CMV-infected HUVECs. The red stain identifies HLA-I stained with the mAB W6/32 and the blue stain identifies CMV-infected cells stained with the mAB ENP. Original magnification 40X.

Figure 29 - Histogram of CMV-infected HUVECs dual stained for CMV and HLA-I. Degree of HLA-I staining plotted on the X-axis, proportion of cells CMV- or CMV+ represented by shading and frequency plotted on Y-axis.
Conclusions

These results show that cytoplasmic as well as surface HLA-I decreases with CMV infection. This result was consistent for both fibroblasts and endothelial cells. These results also show that the down-regulation seen is in the CMV-infected population. The small populations that show upregulation within these cultures represent a non-infected subset of cells, probably responding to the indirect effects of the infected cells.

Therefore, the decrease in HLA-I on the surface of cells cannot be explained by an internalization of surface HLA-I or by a lack of transport to the surface of HLA-I. In either of these cases it would be expected that the cytoplasmic HLA-I would either increase or stay the same in the infected cells.
HLA-I Free Chain Expression

Hypothesis

A possible mechanism for downregulation of surface HLA-I is by prevention of HLA-I to assemble. Functional HLA-I consists of heavy chain, $\beta_2$-microglobulin, and a potential antigen. It possible that the HLA-I gene is transcribed and translated, but not assembled. The flow cytometry and immunohistochemical data shown is based on the binding characteristics of W6/32. This antibody is only capable of detecting intact HLA-I complex consisting of both the heavy chain and $\beta_2$-microglobulin. One possible explanation then, for the lack of observed HLA-I in infected cells, is that the virus interferes with the assembly of $\beta_2$-microglobulin with the HLA-I heavy chain.

The testable hypothesis then would be that HLA-I is translated but does not assemble secondary to CMV infection. To test this hypothesis, we used an antibody developed by Ploegh et. al. which is capable of binding to both intact HLA-I as well as the free heavy chain. It would be predicted that HLA-I heavy chain (hcHLA-I) would be present in normal or increased amounts in infected cells.

Methods

Immunohistochemistry was used to test this hypothesis. HLA-I is not transported to the plasma membrane until after
it assembles; therefore it would be expected that unassembled heavy chain would only be present in the cytoplasm. Flow cytometry of intact cells would thus only identify the heavy chain found assembled on the surface. This would be the same result found using W6/32.

Both fibroblasts and endothelial cells were infected as described in the experiments above. Cytocentrifuge slides were then prepared and fixed in acetone. These slides were then reacted with mAB HC10 as described in Appendix A. HC10 was obtained from Plough as a hybridoma. These cells were grown as described in Appendix A. Antibody was obtained as supernatant harvested from growing cultures. It was used as either an unprocessed supernatant or was concentrated by using a Minicon fluid concentrator. For quantification, one hundred cells were counted using the 0-3 scale described previously with W6/32 staining.

**Results**

**Fibroblasts**

Cultures of MRC-5 fibroblasts were grown to confluence and then infected with 10,000 pfu of VHL-F for 48 and 72 hours. This inocula and time points was based on previous results which showed maximal downregulation with both flow cytometry and immunohistochemistry using the monoclonal antibody W6/32.
Cells were harvested by trypsinization and infection confirmed by immunohistochemistry using ENP and the down regulation of surface HLA-I confirmed by flow cytometry using antibody W6/32. Supernatant from HC10 cultures was used to stain non-infected control and cells infected for 48 and 72 hour. Non-specific staining was assessed by staining with an isotypically matched antibody (L26), and fresh media was used as a negative control.

The following figures show photomicrographs of non-infected control cultures (figure 35) as well as those infected for 48 (figure 36) and 72 hours (figure 37). The staining seen in the non-infected control culture (figure 35) is assumed to be the normal pattern for free chain expression. Little variability is seen between the individual cells. It is possible to see both cytoplasmic as well as membrane-associated binding of the antibody.

Figure 36 is a photomicrograph of HC10 immunohistochemistry 48 hours after CMV infection. Flow cytometry showed a 42% decrease surface expression using the antibody W6/32. In this slide we see upregulation of the HLA-I heavy chain.

At 72 hours, flow cytometry showed a 70% decrease in surface expression of HLA-I. In contrast, HC10 reactivity (figure 37) shows a pronounced increase. This is seen mostly in the cytoplasm with distinct perinuclear staining. The staining is not nuclear as would be expected with non-
specific binding of the antibody. The negative controls with media and L26 showed no staining (results not shown).
**Figure 30** - Photomicrograph of non-infected MRC-5 cells. Stained by immunohistochemistry method using antibody HC-10. Original magnification 40X. Control for Figure 31.

**Figure 31** - Photomicrograph of MRC-5 cells infected for 72 hours with 10,000 pfu of CMV. Stained by immunohistochemistry with mAB HC10. Original magnification 40X.
Cell counts were performed using the 0-3 scale explained earlier. The counts were done in triplicate. The results are graphed in figure 38 below.

![Histogram of HC10 staining in MRC-5 cells infected with CMV for 0, 48 and 72 hours. Time plotted on X Axis with shading representing intensity of staining on a 0-3 scale. Percent in each category plotted on the Y Axis.](image)

**Figure 32** - Histogram of HC10 staining in MRC-5 cells infected with CMV for 0, 48 and 72 hours. Time plotted on X Axis with shading representing intensity of staining on a 0-3 scale. Percent in each category plotted on the Y Axis.

These results show a marked increase in hcHLA-I at both 48 and 72 hours. This staining was seen most prominently in the Golgi region of the cells. At 72 hours the decrease in surface expression by flow cytometry was almost 70%, whereas HC10 showed upregulation on virtually all the cells. This supports the theory that CMV does not interfere with transcription or translation of the HLA-I genes. CMV must
therefore interfere with HLA-I surface expression at a point after translation.

**Endothelial Cells**

Similar immunohistochemistry was performed on HUVECs as was done on the above fibroblasts. A non-infected culture acted as the control and a culture infected for 8 days and 10 days acted as the experimental. The expression of HLA-I was normal (figure 40), decreased by 75% (figure 41), and decreased by 97% (figure 42), as measured by flow cytometry using the antibody W6/32.
Figure 33 - Photomicrograph of non-infected HUVECs. Stained by immunohistochemistry method with mAb HC10. Original magnification 40X. Control for Figure 34.

Figure 34 - Photomicrograph of highly CMV-infected HUVECs. Stained by immunohistochemistry with mAb HC10. Original magnification 40X.
Results showed faint but even expression of hcHLA-I in the non-infected control. However, hcHLA-I was markedly increased for the infected cultures. Figure 41 had a 76 percent decrease in surface HLA-I as measured by flow cytometry using the antibody W6/32. However, most of the cells in the culture show increased expression of HLA-I using the antibody HC10. This is even more dramatic in Figure 42 where there was a 97% decrease in intact surface HLA-I expression by flow cytometry and virtually every cell has increased expression when compared to the control when looking at the cytoplasmic heavy chain of HLA-I.

HC10 binding was assessed on 100 cells from each slide and assigned a number on a scale from 0-3 for expression of free chain. Average results are shown in figure 35 below. These results are similar to that seen with the fibroblasts. In cells with higher levels of infection, increased expression of HLA-I heavy chain is seen in the cytoplasm. These results were found to be statistically significant.
Figure 35 - Histogram of HC10 staining of non-infected and CMV infected HUVEC's. Control bars are labeled CMV- whereas the middle and right cluster of columns are 75% and 97% CMV infected as shown. Percent in each category is plotted on the Y axis.

Conclusion

These results support the hypothesis that the downregulation in surface expression of intact HLA-I is caused by a failure in post-translational assembly of the HLA-I heavy chain and β2-microglobulin. They would further support a hypothesis that these cells are still sensitive to interferons since there appears to be upregulation in the expression of HLA-I free heavy chain. This would be expected in a population of virally infected cells because of the
release of interferons leading to increased transcription and translation of the HLA-I genes.
HLA-I mRNA Expression

Hypothesis

The hypothesis that surface HLA-I expression is downregulated by a posttranslational mechanism can be further tested by looking at mRNA levels in infected populations of cells. If a CMV-infected population of cells produces interferons and increases expression of hcHLA-I, but fails to assemble these into functional intact HLA-I surface molecules, it can be predicted that the mRNA levels for HLA-I would be increased. This hypothesis was tested by performing northern blot analysis on infected and non-infected cells. In this way, the level of HLA-I mRNA in these cultures could be assayed.

Methods

Cultures of MRC-5 fibroblasts or HUVECs were infected as previously described. Fibroblasts were infected with 100, 1000, or 10,000 pfu of VHL-F CMV for 24, 48 or 72 hours. Total cellular RNA was isolated as described in the Appendix. Northern analysis was then performed using the method of Manniatis as described in the Appendix. An aliquot of cells was taken from the original culture and flow cytometry was performed to determine the change in surface expression and/or immunohistochemistry using mAB ENP.
was performed to determine the level of CMV infection.

Endothelial cells were grown to confluence and infected with VHL-E. These were maintained and checked daily for the progression of the infection. Cells were harvested at varying times after infection when the desired level of infection as determined by changes in cellular morphology was achieved. Some cultures were stimulated with γ-interferon to assess whether normal effects on HLA-I mRNA were still effective.

**Results**

**Endothelial Cells**

The first northern blots were performed to establish the validity of the assay and, in the same experiment, to look at HLA-I mRNA levels in cultures of HUVECs with low levels of CMV infection. γ-IFN stimulation was used to establish that the assay reacted appropriately as established by the literature for non-infected cultures. It would be expected that there would be strong upregulation of HLA0-I mRNA with stimulation by γ-IFN.

Figure 41 shows a photograph of the exposed film from a northern blot performed on four cultures of endothelial cells. Lane A is an non-infected culture of HUVECs. Lane B is an non-infected flask of HUVECs stimulated with 200 units of γ-IFN for 72 hours. Lane C is a CMV-infected of HUVECs.
with no other treatment. Lane D is a culture of HUVECs infected with CMV for 3 days and then stimulated for 72 hours with $\gamma$-IFN. The cultures shown in lanes A and B showed no CMV infection as determined by ENP immunohistochemistry. Lane C showed 5% positive cells whereas the cells from lane D showed 10% positive for CMV.
Figure 36 - Northern blot of HUVEC cells. Lane A: Non-infected, Lane B: Non-infected and stimulated with γIFN, Lane C: CMV infected, lane D: CMV infected and stimulated with γIFN.

Figure 37 - Results of digitized northern blot shown in figure 39. Lane A: Non-infected HUVECs, Lane B: Non-infected, IFN-treated HUVECs, Lane C: CMV-infected HUVECs, Lane D: CMV-infected, IFN-treated HUVECs.
It is clear, by looking at lanes A and B, that a reasonable level of HLA-I mRNA is detected by this assay and that it responds appropriately to stimulation by γ-IFN. This validates the assay based on the literature. It is also clear that a low level of CMV infection does not decrease HLA-I mRNA levels. In this particular experiment it appears that there is a moderate increase in HLA-I mRNA. In this experiment HLA-I protein expression was not assayed so it is unknown whether this increase in HLA-I mRNA correlates to a downregulation in HLA protein.

The following figure shows the results of digitizing the film shown in figure 39. The absorbance was corrected by total ribosomal RNA staining with EtBr as described in the Appendix. These digitized results confirms a moderate increase in HLA-I mRNA. Treatment with IFN increased mRNA levels in both the non-infected and CMV infected HUVECs. CMV infection increased mRNA in both the non-treated and the IFN treated cultures.

The next experiment was designed to determine whether a culture with a higher level of infection, resulting in a significant decrease in surface HLA-I protein, had an increase or decrease in HLA-I mRNA. To decrease the influence of the mRNA from uninfected cells, an attempt was made to infect as large a percentage of cells as possible. The percentage of infection was assayed by ENP
immunohistochemistry. The level of HLA-I on the surface of these cells was measured by flow cytometry.

The infected culture showed 74.3% of the cells positive for the ENP antigen with no cells staining in the control culture. The control culture had a net mean fluorescence of 112.3 compared to 82.8 in the CMV infected culture. This is a 26% decrease in surface expression. Figure 41 shows the resulting film from the northern blot.
**Figure 38** - Northern blot of non-infected HUVECs on the left and CMV infected HUVECs on the right. 74% of the infected culture was positive for ENP.

**Figure 39.** Results of digitizing the northern shown in Figure 37.
This northern blot shows an increase in the level of HLA-I mRNA despite a significant decline in HLA-I surface expression in a culture with high levels of CMV infection. This film was digitized and the results shown in the following figure. It was found to have a 62% increase in HLA-I mRNA. This gives strong evidence that CMV does not interfere with the transcription of HLA-I mRNA. This particular experiment did have more than 25% of the cells not infected with CMV as assayed by immunohistochemistry and the decrease in class-I protein was only 25%. Therefore, the increase in HLA-I mRNA could be explained by a large increase in HLA-I mRNA in the 25% uninfected cells.

Therefore, this experiment was repeated and the infection allowed to progress further. The following figure shows a northern from culture with a 76% decrease in surface HLA-I with greater than 90% of the cells infected with CMV (figure 43). Figure 44 shows the results of digitizing the film in figure 43. This reconfirms as did all northerns performed on endothelial cells that CMV infection leads to increases in HLA-I mRNA.
Figure 40 - Northern blot of non-infected HUVECs on the left and CMV infected HUVECs on the right. 92% of the cells positive for ENP in the infected culture with a 73% decrease in surface HLA-I.

Figure 41. Results of digitizing northern shown in Figure 40.
Fibroblasts

Similar experiments were performed in fibroblasts as have been described in endothelial cells. The first experiment was designed to look at early time points after CMV infection. 1000 PFU per flask of VHL-F was used to infect the MRC-5 cells for 12, 24, 36, and 48 hours. Level of infection was determined by flow cytometry. RNA was isolated and northern blot analysis performed. The northern blots were digitized and corrected for differences in loading by digitizing a photograph of rRNA staining. The experiment was performed in duplicate.

A photograph of the northern is shown in figures 41. Lane A is the non-infected control whereas lanes B-E are 12, 24, 36, and 48 hours post infection respectively. Both experiments had considerable variability in the loading of total RNA in the lanes. Therefore, the corrected data in figure 42 shows considerable differences from the raw film. On the same graph is plotted the change in HLA-I surface protein for the same cultures.
Figure 42 - Northern of CMV-infected MRC-5 cells. Infected with 1000 pfu of VHL-F. Lane A: 0 hours, Lane B: 12 hours, Lane C: 24 hours, Lane D: 36 hours, Lane E: 48 hours. Probed with HLA-B7.

Figure 43 - Line graph of HLA-I flow cytometry data in the squares plotted on the left y-axis and HLA-I northern analysis in the diamonds plotted on the right y-axis. X-axis is time in hours.
These figures show a decrease in protein expression at 36 hours with a slight recovery at 48 hours. However, the mRNA expression goes up at 12, 24, 36 and 48 hours. The increase at 48 hours is very significant. The increase in surface expression is consistent with that shown in the flow cytometry data previously shown (see figure 16). This data shows that an overall decrease in protein expression is associated with an overall increase in mRNA expression. This is similar to the data shown for HUVECS in the steady state system. It also shows that the increase in mRNA begins as early as 12 hours after infection. At that time point, the surface expression is already decreasing. The largest increase in mRNA correlates with the upswing in surface expression seen at 48 hours. It is important to remember, in these experiments, that not all of the cells in the flask are infected. Cell counts reveal that only 50 percent of the cells are infected at 48 hours. Therefore the increase in surface expression could be occurring in the infected cells, the non-infected cells, or both. As shown before, the larger the inoculum of virus, the less the increase in surface expression at 48 hours.

The following experiment was performed using 10,000 pfu of virus for 24, 48 and 72 hours. These conditions give maximal downregulation of surface HLA-I. The flow cytometry data is plotted as the squares figure 49. This shows a steady decrease over the 72 hour period to almost zero.
Figure 48 shows the northern blot from these same cells. Despite the decrease in surface expression shown by flow cytometry, the signal from the HLA-I mRNA increases from the non-infected control and continues to be significantly upregulated out to 72 hours. The digitized, corrected values are plotted as the diamonds on the same graph as the flow cytometry data (Figure 49).
Figure 44 - Northern of MRC-5 cells infected with 10,000 pfu of VHL-F. Lane A: non-infected control Lane B: 24 hrs., Lane C: 48 hours, Lane D: 72 hours.

Figure 45- Line graph of HLA-I flow cytometry data in the squares plotted on the left Y-axis and HLA-I northern analysis in the diamonds plotted on the right y-axis. X-axis is time in hours.
Conclusion

In fibroblasts and endothelial cells, CMV-infected cultures never showed decreases cytoplasmic levels of HLA-I mRNA. On the contrary, cultures which showed marked decreases in HLA-I surface and cytoplasmic protein showed increases in levels HLA-I mRNA. In cultures which had populations with both CMV-infected and non-infected cells, it is possible that the increase seen is due to the effects of IFN on the non-infected culture. However, when the cultures of both fibroblasts and endothelial cells were near 100% infected with near 100% decrease in surface HLA-I, there were still normal amounts of HLA-I mRNA present.

This data requires rejection of the hypothesis that the down-regulation of surface HLA-I in CMV infection is due to a decrease in HLA-I levels either through transcription or by a decrease in the half life of HLA-I mRNA.
DISCUSSION

Surface Expression

The results shown here clarify the debate on the effect of CMV infection on HLA-I expression. The system is more complicated than has been previously described. These results show that in a population of fibroblasts or endothelial cells, the surface expression of HLA-I decreases with the increasing level of CMV infection. However, at low levels of infection, the indirect effects on the non-infected population can lead to upregulation of both surface and cytoplasmic HLA-I. This is most probably due to the release of cytokines by the infected population.

These results are in agreement with other investigators although the interpretation varied. This was due in large part to either a poor model or the inability to discriminate between the behavior of a population of cells from the behavior of individual cells.

The results presented here give evidence that CMV-infected cells downregulate their surface expression HLA-I. This is shown in the flow cytometry data of highly infected cultures of both HUVECs and fibroblasts. Both the histograms
all the cells, as well as a decrease in the mean fluorescence when the cultures were greater than 90% infected. In fibroblasts this effect was found to be dose and time dependent. In endothelial cells, a positive correlation was found between the degree of infection and degree of decrease in HLA-I.

Conclusions can be made about the behavior of individual cells since virtually all the cells in the population were infected and virtually all the cells in the population showed downregulation.

van Dorp had looked at HLA-I expression in CMV-infected HUVECs and found that HLA-I increased. They used the monoclonal antibody W6/32 and a monoclonal antibody to β2-microglobulin. Their assays consisted of a radioimmunoassay and flow cytometry. In both assays they were measuring surface expression of intact HLA-I. The level of CMV infection was assayed by immunohistochemistry using an antibody to an early CMV protein. Therefore, this model was essentially identical to ours except for the strain of CMV virus used.

The authors stated that the level of CMV infection was found to be as high as 10% of the cells. They observed a two fold increase in mean surface HLA-I as measured by RIA. Flow cytometry showed that 10% of the cells increased in their surface HLA-I. They concluded that since the amount of increase in HLA-I was proportional to the amount of observed
infection, that this was a direct effect of infection and not due to soluable factors. They were not able to identify whether the subpopulation of cells which were infected were the same subpopulation which was showing an increase in surface HLA-I.

An important difference in methods relates to the viral strain used to infect the endothelial cells. They used CMV strain AD 169 and were only able to achieve infections of up to 10%. They also used an early time-point of 48 hours post-infection at which to perform these assays. Our data would predict similar results for these conditions. At low levels of infection, less than 10%, we would frequently see upregulation in a subpopulation of cells. However, in our experiments, dual stain immunohistochemistry showed that the upregulated population is not the CMV-infected population. Also, at high levels of infection, and late time-points, when most of the cells in the population were infected, only decreases in CMV surface expression were found in endothelial cells. Therefore, their results are consistent with ours, although their conclusions are not.

The observed upregulation in HLA-I in cells with low levels of infection can be explained by the known effects of interferons. If the CMV-infected cells produce interferons in response to viral infection. This in turn would lead to an increase in surface HLA-I on the non-infected cells in the same culture. Cells infected with a virus, including CMV, produce α and β interferons. It is also known that α and
\( \beta \) interferons cause upregulation of surface HLA-I in both fibroblasts and endothelial cells. With the above information one would predict at low levels of infection a few cells would show downregulation of HLA-I and many cells would show upregulation of HLA-I. At moderate levels of infection you would see two populations of cells, those infected cells with decreased HLA-I and those non-infected, interferon-stimulated cells with upregulated HLA-I. In highly infected cells it would be predicted that virtually all of the cells would show downregulation.

A further prediction can be made on the time course of the upregulation. It is known that the production of, and response to, the interferons should take around 48 hours. All of these predictions were found to be true in our time/dose studies of the fibroblasts. With 1000 pfu inocula, an increase in HLA-I is seen at 48 hours in fibroblasts.

A dual peak in HLA-I expression was seen by Hosenpud et. al. in smooth muscle cells infected with CMV. This dual peak is essentially identical to the one shown in fibroblasts by this work. The upregulated peak showed a 3 fold increase in the number of HLA-I antigens whereas the downregulated peak had a tenfold decrease in HLA-I. No conclusion could be reached about the composition of these two peaks, time course studies were not performed, and no attempt was made to identify which cells were CMV positive.

Hosenpud also reports experiments on endothelial cells but was unable to achieve infection levels greater than \( \%10 \).
He found no significant change in HLA-I expression in these cells. Again, this would be consistent with our data at low inocula.

Because of the confusion in the literature dealing with whether HLA-I protein is increased or decreased, not much work has been done on the mechanism of these changes. One author has approached this subject. He proposed a mechanism of downregulation which is due to the expression of the Class I homologue by CMV infected cells. He showed that cells transfected with both the class I homologue and β2-microglobulin express this viral protein on their surface associated with β2-microglobulin. He also demonstrated that HLA-I mRNA levels did not decrease in CMV infected cells. Therefore, he concluded that the mechanism of HLA-I downregulation was the binding of β2-microglobulin by the class I homologue which would prevent HLA-I from binding it and being transported to the surface.

This work is unique in that it was able to demonstrate that hcHLA-I is indeed present in infected cell. Therefore, it is possible to conclude that CMV infection does not interfere with the transcription or translation of the HLA-I genes. This supports class I homologue as a potential mechanism for the downregulation of HLA-I surface expression.
Other models exist for the interference of virus' with the expression of HLA-I. In all of these models, the decrease in HLA-I expression results in the ability of the virus to evade detection by the immune system and persist. Adenovirus type II is the only other virus which has been shown to downregulate HLA-I by a post-translational mechanism. It has been demonstrated that a protein coded by the viral genome complexes with HLA-I which prevents its surface expression.

It has not been shown whether a similar mechanism could be involved with CMV. No viral proteins have been demonstrated to bind or complex with HLA-I. However, a more indirect mechanism is possible as described by XXX which involves complexing with β2-microglobulin which then prevents expression of HLA-I by lack of β2-microglobulin protein.

This hypothesis could be further tested by two transfection experiments. In the first, the class I homologue should be able to decrease surface expression of HLA-I by transfecting cells with this protein only. This would lead to all the β2-microglobulin being sequestered in the cell. The second experiment would be to try to reverse the effect of CMV infection by transfecting cells with β2-microglobulin and overexpressing this gene. In this way
surplus β2-microglobulin would be available to allow transport of normal HLA-I to the surface.

Epstein-Barr Virus and Herpes simplex virus have also been shown to decrease surface expression of HLA-I but no mechanism has been determined to this time point. The use of northern analysis and HC10 antibody should help to determine whether the mechanism is pre-transcription, pre-translation or post-translation. By following the methods in this dissertation many possible mechanisms can be eliminated and then the search narrowed for the correct factor.

**Transplantation**

The association between CMV infection and allogeneic graft rejection has been demonstrated by many investigators. This research has not attempted to address whether the association between CMV and graft rejection is real. The goal was to examine a possible mechanism for this association.

One possible explanation for these results is that the association between CMV infection and graft rejection is not a cause-and-effect relationship. The poor outcomes in patients with CMV infection may be due to the clinician's intervention, leading to treatment of the infection by decreasing the level of immunosuppression. This would then lead to increasing possibility of rejection.

The results found in this research support the work by some investigators but refute the work of others. Looking
closely at the experimental design and results of these other experiments helps to explain the differences in findings.

**Viral Persistence**

Many viruses have evolved mechanisms by which they can evade immune destruction, and many immune systems have developed mechanisms by which to defeat them. A particular virus-host relationship can perhaps best be judged by overall outcomes. A successful virus could be defined as one which propagates within a host without killing the host before the virus is able to spread to another host. In these terms, the persistent virus is perhaps the most successful of all since these viruses are able to remain in a host indefinitely and still be able to spread to other individuals.

Oldstone has discussed possible mechanisms of viral persistence and includes modification of HLA-I, modification of HLA-I, changing of T-cell function, decreasing the presentation of surface antigens etc. With this in mind it would be predicted that a virus which was successful in persistence may do so by altering the surface expression of class I. Examples can be found in the review of the literature. Herpes virus is perhaps the best known of the persistent viruses. It goes through phases of activation and latency. It could judged a relatively "successful" virus since it infects nearly 100% of the population.
Researchers have shown that Herpes virus downregulates class I in cells which they have infected. What has not been looked at is whether this downregulation is functionally significant and allows the infected cell to evade immune surveillance.

Another important point of discussion is the research done by other investigators looking at class I expression in CMV-infected cells. Some research has been done on very similar models as described here with different results. Other investigators have found similar results as ours but have interpreted them differently.

This corresponds to the golgi apparatus. HLA-I protein which does not associate with \(\beta_2\)-microglobulin is unable to be transported beyond the golgi apparatus according to Peters et al.\(^{101}\)
APPENDIX A: Tissue Culture Techniques

Human Umbilical Vein Endothelial Cell Culture

Isolation of primary cultures

HUVECs were isolated from umbilical cords using a modification of the Jaffe method. Cells were established as primary cultures from umbilical cords obtained within 48 hours of delivery. The umbilical vein was then cannulated and flushed with several volumes of phosphate-buffered saline (PBS) until all traces of blood were removed. The PBS was then removed and the cord was filled with a solution of 0.1% collagenase in PBS for 30 minutes. The collagenase solution containing endothelial cells was then removed and the cord rinsed with 2 volumes of PBS which was combined with the collagenase solution. This was then centrifuged at 1000 rpm for 10 minutes to pellet the endothelial cells. This pellet was then resuspended into endothelial cell growth medium (ECGM). This medium was composed of 76.8% (v/v) M-199, 19% (v/v) fetal bovine serum, 2% (v/v) Hepes buffer, 2% (v/v) heparin/endothelial growth factor (ECGF), and 0.2% (v/v) penicillin (5.97 gm/100ml) streptomycin (10 gm/100 ml PBS). ECGF was
prepared from fresh whole bovine brain according to the method of Maciag et al.\textsuperscript{104} M199 was obtained from Gibco and was modified with Earl's salts, L-glutamine, sodium pantothenate.

Cell counts were performed on the resuspended endothelial cells and the cells seeded on fibronectin coated plastic culture plates (Corning Glass Works, Corning, New York) and incubated at 37°C in 95% room air/5% CO\textsubscript{2}. Cells were harvested by trypsinization with 0.01% trypsin/0.02% EDTA after reaching confluence and then passed into larger flasks.

**Characterization**

All cells were characterized by immunohistochemistry to confirm the identify of the cell type and ensure the lack of any contaminating cell types. This was done between passages three and four. Von Willebrand Factor was used as a positive marker of endothelial cells. In addition, cells were stained for leukocyte common antigen (LCA), a monocyte-specific marker (LeuM5), and a B-cell marker (L26) to exclude the presence of contaminating leukocytes.

Staining was performed by the indirect immunoperoxidase technique described in Appendix B. All cultures stained positive for Von Willebrand Factor and negative for all other makers.
**Maintainance of cultures**

Cells were grown in the same EGM described above. Cells were plated into plastic culture flasks coated with fibronectin. HUVECs were kept in a humidified incubator with 95% air/5% CO\(_2\). Cells were fed every 2-3 days by aspirating off the media and replacing it with fresh media.

Cells were passaged when confluent. The media was removed and the cells were washed twice with PBS. Three milliliters of 0.01% trypsin/0.02% EDTA was placed onto a 150cm\(^2\) culture flasks. During the next two to three minutes the cells would release from the flask. They were then collected by adding a 10 fold surplus of media and placing the cells into an appropriate sized flasks. Cells were used between passages four and eight. Cells were passaged to an appropriate sized flasks for the described experiment.
Fibroblasts Cell Culture

The fibroblast strain used for these experiments was MRC-5 (ATCC #CCL 171). This is an embryonal lung fibroblasts isolated from a human male. They were used between passages twenty-five and thirty for all described experiments.

Cells were cultured in B-media which consisted of MEM supplemented with essential amino acids, nonessential amino acids, vitamins, sodium pyruvate, and 10% fetal calf serum. Cells were harvested with 0.01% trypsin/0.02% EDTA after washing twice with PBS. They were then diluted into an appropriate volume of media. Cells were subcultured at a ratio of 1:2 to 1:5.
APPENDIX B: Viral Techniques

The following strains of CMV virus were used in the described experiments:

AD169 - Obtained from American Type Culture Collection VR-538. It was propagated in NHDF cells.

VHL-E - Strain obtained as a clinical isolate and propagated for 15 passages in NHDF cells.\(^{105}\)

VHL-F - Strain obtained as a clinical isolate and propagated for 15 passages in HUVECs.\(^{106}\)

Virus were propagated in the above indicated cell types and harvested when >90% of the cells exhibited cytopathology. Cells were washed twice with PBS. Cells were released by the addition of 0.01% trypsin/0.02% EDTA (1.5 ml of each reagent per 150 cm\(^2\) flask) followed by incubation for 5 minutes at 35.5°C. Cells were then resuspended in storage medium consisting of 40% MEM, 10% fetal bovine serum, and 50% sucrose phosphate buffer (188 mM sucrose, 12 mM K\(_2\)HPO\(_4\), 7.5 mM KH\(_2\)PO\(_4\)), transferred to 1.8 ml cryovials (Nalgene), and rapidly frozen over liquid nitrogen. Frozen stocks were stored in liquid nitrogen vapors.

Concentrations of virus stocks, in terms of plaque forming units (PFU)/ml, were determined by plaque assay titration on NHDF monolayers in six-well culture plates.
(Corning) according to a protocol based on the method of Wentworth and French.\textsuperscript{107} Titers of each viral strain were derived from counts of 10 replicate wells.
APPENDIX C: Immunohistochemistry

Slide preparation

Slides were prepared by cytocentrifugation. Cells harvested from cultures were suspended in a solution of culture media at a concentration of 100,000-300,000 cells/ml. 200 ul of this cell suspension was placed into each well of a Shandon Cytospin 2 (Shandon Inc., Pittsburgh, PA) cytocentrifuge. The cytocentrifuge was spun at 1000 rpm with low acceleration for five minutes. The slides were air dried and fixed by placing in cold acetone for five minutes. This resulted in a glass microscope slide with a 6mm circle of cells. These cells had circular morphology without significant damage to the cells. These slides were stored at -20°C until ready for staining.
Primary Antibody

Slides were rehydrated in TBS (0.05M Tris pH-10.6/0.9%NaCl) for five minutes and then blocked in horse serum diluted 10:1 in TBS for 15 minutes. One of the following primary antibodies was diluted into antibody diluting solution (ADS) and then placed onto the cells and incubated for 30 minutes at 37°C in a humidified chamber. The concentrations at which the antibodies were used was determined experimentally to give the highest possible specific binding and the lowest possible background. ADS is made by mixing 10 mls of Tris-Hcl buffer (0.5M, pH 7.6) 10 mls of 2% Gelatin (Sigma) in 0.9% NaCl and 80 mls of 0.9% NaCl.

HLA-I

HLA-I was detected by the monoclonal antibody W6/32. It was first produced and characterized by Barstable et. al. W6/32 is a mouse monoclonal antibody, with a IgG2a heavy chain and a kappa light chain. It binds to intact HLA I complex at a public domain. It requires an assembled heavy chain, β2-microglobulin, and antigen complex to bind. It binds equally well with HLA-A,B or C. This antibody detects HLA-I on nucleated cells in all normal human tissues. It has been used to demonstrate both increases and decreases in HLA-I in pathologic conditions.
For immunohistochemistry, W6/32 was obtained from DAKO at a concentration of 94 ug/ml. It was then diluted to 1:100 for use. Leu26 was used as an isotypic control to for non-specific binding.

For flow cytometry, antibodies from two manufactures were used. The same DAKO W6/32 antibody was used in the indirect method. It was diluted 1:20. A secondary FITC labeled antimouse antibody was then used to label the cells.

A FITC labeled W6/32 was obtained from Olympus to perform direct labeling of cells for flow cytometry. The negative control was a non-specific IgG2b FITC labeled molecule also provided by Olympus. This method provided lower backgrounds and more intact cells due to the decrease need for manipulation. This antibody was used neat as described in the section on flow cytometry.

B-Cell

A B-cell marker was used as a non-specific idiotypic control for ENP, W6/32 and HC10. This antibody is termed L26 and was obtained from DAKO. It is a member of the CD20 family and is specific to an epitope present in B-cells. This epitope is intracellular and resistant to formalyn fixation. It is specific to B-cells with no binding found on other leukocytes or other tissues. It was received from DAKO at a concentration of 156 ug/ML and used at a dilution such that the protein concentration was the same as the primary antibody for which it was acting as a control.
Von Willebrand Factor

Von Willebrand factor was identified by a mouse monoclonal antibody designated F8/86. It was obtained from DAKO at a concentration of 157 ug/ml. This antibody reacts to human Von Willebrand factor and can be used to identify endothelial cells and megakaryocytes.115 This antibody is an IgG1 subclass. F8/86 antibody was used at a dilution of 1:600 to stain culture slides of endothelial cells. This was done to characterize new cultures as a positive marker of endothelial cells. This is described in greater detail in the section on endothelial cell culture.

CMV ENP

An antibody to CMV early nuclear protein was obtained from Dupont. It is a mouse monoclonal antibody of the IgG2A isotype. It is directed to a nuclear protein produced early in infection. It has some cross reactivity to some EBV antigens by none to normal huma tissues. It is obtained at a 100 ug/ml concentration. This antibody was used in immunohistochemistry at a concentration of 1:100. This gave clean staining of just the nucleus of infected cells.

Human Leukocyte Common Antigen

Dako-LC is a mixture of two monoclonal antibodies specific for LCA present on most human leukocytes.116 It identifies a determinant termed CD45.117 This antibody was used to characterize cultures of endothelial cells to
demonstrate the absence of contaminating leukocytes. It was used at a concentration of 1:100.

**Leu-M5**

S-HCL-3 is a monoclonal antibody obtained from Becton-Dickinson. It is used to identify the antigen Leu-M5 also termed CD11.\(^{118}\) This antibody is mouse monoclonal antibody of the IgG2b subtype. It stains macrophages and cells of macrophage lineage including histiocytes, Kupffer cells and alveolar macrophages. It was used in immunohistochemistry to characterized endothelial cell cultures to demonstrate the absence of contaminating macrophages.
Secondary Antibody

Slides were washed three times in Tris Buffered Saline (TBS) to remove all non-specific binding of the primary antibody. Slides were then dipped one time into a 1:10, horse serum:TBS solution and then secondary antibody placed onto each slide. The secondary antibody was a horse anti-mouse polyclonal antibody with a biotin conjugate which was obtained from Vector. This antibody reacts with all IgG subclasses. This antibody was used at a 1:200 dilution into ADS. The slides were incubated for 15 minutes at 37°C.
**Avidin-Enzyme**

Slides were again washed three times with TBS and then a 1:500 dilution of an avidine enzyme linked conjugate was placed onto each slide. The enzyme consisted of either horseradish peroxidase or alkaline phosphatase. Both these enzymes were obtained from Vector. The slides were allowed to incubate for 15 minutes at 37°C for 15 minutes in a humidified chamber and then washed 3 times with TBS. The above concentrations, which were experimentally determined to yield maximum specific and minimum background staining. These were incubated for 45 minutes at 37°C in a humidified chamber. Slides were then washed three times in TBS and then incubated with biotinylated, affinity-purified, horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 15 minutes at 37°C in a humidified chamber. Slides were then again washed three times in TBS and incubated with avidin D either linked to horseradish peroxidase or linked to alkaline phosphatase (Vector Laboratories, Burlingame, CA) for 15 minutes at 37°C in a humidified chamber. The slides were then washed in TBS and developed by the appropriate technique described below.
Horseradish Peroxidase Reaction

The method used is an indirect immunohistochemical technique. A solution of 0.3% 32-amino-9-ethylcarbazole in dimethyl-formamide (AEC) (Sigma) was diluted ten fold with acetate buffer (0.02M, pH 5.2). This was filtered through filter paper and then 40 ul of 3% hydrogen peroxide was added to the AEC immediately prior to use. Slides were soaked in acetate buffer for five minutes and then developed in the AEC until the desired color change had occurred. Slides were washed for 10 minutes in running tap water and then were counterstained with Gill-3 hematoxylin and coverslipped with glycerine jelly.
Indirect Alkaline Phosphatase Reaction

Alkaline phosphatase was conjugated to the avidin molecule in the avidin biotin reaction. After rinsing with TBS, a substrate solution was placed onto the slides which were then allowed to develop in the dark for 10-30 minutes. The substrate was obtained from Vector and prepared according to the manufacturer's instructions. No counterstain was used.
Double Labeled Indirect Reaction

Double staining was used to identify the HLA-I status and whether a cell was positive for CMV at the same population of cells. The slide was reacted with W6/32 first as described previously. The secondary antibody linked to biotin and the avidin-horseradish peroxidase were then incubated on the slide and washed as previously described. AEC was used as the substrate for the color reaction. After the appropriate color reaction was achieved, the slides were washed with TBS and then blocked with an Avidin/Biotin blocking kit (Vector). Each blocking agent was left on for 10 minutes and then thoroughly washed with TBS.

The second antibody was then placed which consisted of ENP. This was incubated as described before. In this instance, the avidin was linked to alkaline phosphatase. The appropriate substrate was then placed on the slide, and the reaction allowed to progress to completion. No counterstain was used.
APPENDIX D: Flow Cytometry

An indirect immunofluorescence method was used. The primary antibody used is 37WB which is a mouse monoclonal antibody. It is specific to a public domain of class I molecules. It therefore binds to HLA-A, -B, -C. The secondary antibody is a horse anti-mouse molecule conjugated to a FITC molecule. The method involves collecting the cells from the flask by trypsinization. Usually aliquots of the cells are separated for making cytocentrifuge slides or for mRNA isolation.

Cells were harvested by a 1.5 minute exposure to 0.01% trypsin/0.02% EDTA (Sigma) in equal volume at 37°C. ECGM was then added to the flask in excess. Cells were counted, centrifuged into a pellet and then resuspended to a final concentration of $2 \times 10^6$ cells/ml in ECGM. Cells were incubated at 5°C for 20 minutes with primary antibody which consisted of either HLA I or Leu5 which acted as a negative isospecific control. Cells were washed 3 times with PBS and then incubated for 20 minutes at 5°C with the secondary antibody which was horse anti-mouse conjugated to fluorescein. Cells were again washed in PBS and resuspended into 250 ul of PBS. 5000 cells were then analyzed on a 1.5 minute Epics C flow cytometer (Coulter Corp., Hialeah, Fl).
number of positive cells were determined using a logarithmic scale. Mean fluorescence per cell was analyzed on a linear scale.
APPENDIX E: Northern Analysis

RNA Isolation

RNA was isolated using the technique of Chomczynnski and Sacchi\(^{120}\). A T150 flask of either HUVECs or fibroblasts was washed with PBS two times and then treated with 3 mls of Trypsin/EDTA for several minutes until the cells released from the plate. 8 mls of M199 media was then added to a final volume of 11 mls. The solution of mixed gently and then 1 ml was removed for use in immunohistochemistry and or flow cytometry. The rest of the cell suspension was centrifuged at 1000 rpm for ten minutes. The resulting pellet was then suspended 1ml of RNAzol (Cinna/Biotecx, Houston, TX) which is a 50\% phenol/2M guandinium thiocyanate solution. This was then agitated to dissolve all the cells by vortexing 15 seconds. The suspension was then transferred to a 1.5 ml snap top tube and 100 ul of chloroform added to the tube. This was again vortexed for 15 seconds and then cooled for 10 minutes at 4°C. The mixture is then centrifuged at 12,000 rpm for 20 minutes to separate the mixture into an aqueous and organic phase. The aqueous phase was then transferred to a fresh snap top tube. This was usually 500-600 ul of a thick solution. An equal
volume of absolute isopropyl alcohol was added, mixed and placed at -20°C overnight. This resulted in a fine RNA precipitate. The tube was then centrifuged for 30 minutes at 12,000 rpm to pellet the RNA. The pellet was then washed 2 times in 75% EtOH. The pellet was resuspended into 400 ul of TE (10mm Tris pH-8.0, 1mM EDTA). A 40 ul sample of this preparation was then diluted into 1000 ul of TE and scanned on a spectrophotometer (Beckman model DU6) from 290 nm to 240 nm. The scan was performed to analyze the quantity as well as the purity of the RNA preparation. An Ab260 of 1.0 equals a concentration of 40ug/ml. The purity was estimated by taking the ratio of Ab260/Ab280. Ideally this would give a ratio of almost 2.0.
RNA was analyzed by agarose gel electrophoresis using the method described by Maniatis et. al. Gels were cast by mixing 40 mls of diethylpyrocarbonate treated H$_2$O (dH$_2$O), 5 mls of 10X borate buffer, 5 mls of 37% formaldehyde and 0.5g of agarose. This mixture was then heated to boiling for 5 minutes, allowed to cool to 60°C. The gel was poured into a 10 cm by 8 cm electrophoresis chamber. This resulted in a 1% agarose gel with 3.7% formaldehyde.

The gel was submerged in an electrophoresis tank containing 250 mls of 1X borate running buffer. This consisted of 200 mls of dH$_2$O 25 mls 10X borate buffer and 25 mls of 37% formaldehyde. The volume containing 30 mg of total RNA as calculated from spectrophotometry was calculated for each sample. This was transferred to a 1.5 ml snap top tube and precipitated by adding a 1/10th volume of Na·Acetate and a two times volume of 100% ethyl alcohol. This was centrifuged for 30 minutes after one hour at -20°C. The pellet is then dissolved in 8ul of TE, 1.5 ul of 10X Borate Buffer, 5.5 ul of formaldehyde and 15 ul of formamide. This was vortexed for 15 seconds and then heated to 65°C for 5 minutes. 5 ul of tracking dye was then added and each sample was loaded onto the gel. This was ran at 70 volts for 2 hours and then stained overnight in a solution of EtBr in H$_2$O. The gel was then washed in 100 mls of depc H$_2$O for 1 hour. This was then photographed under ultraviolet
light using Polaroid type 667 film. Ribosomal RNA was quantitated by scanning a negative image of this film on a Ambis Optical Digitizer. Both 18S and 27S ribosomal RNA was scanned and the area under the curve added together for an area under the curve in mm$^2$ of total rRNA. This number was then used to correct the absorbance found on northerns.
Blotting

Gels were blotted by soaking the agarose gel in a 5X solution of SSC for 10 minutes and then placing on a platform covered with blotting paper. The gel was covered with Nytran which had been wetted in H2O. Above this was layered two pieces of blotting paper and six inches of cut to size paper towels. 500 mls of 5X SSC was placed below the gel and allowed to wick up to the paper towels above the gel. This progressed overnight. The nytran was then allowed to air dry and baked at 80°C under vacuum for two hours.
Hybridization

The nytran filter was placed into a plastic envelope and wetted with 2.5 mls of depc H$_2$O. 2.5 mls of prehybridization solution was then added (Sigma). This consisted of 10X SSC, 10X Denhardt's, and 200ug/ml of DNA. This was incubated at 45°C for 4 hours. At that time the labeled probe was boiled for 5 minutes and added to the bag and incubated at 65°C overnight.

The nytran was removed and washed in 1X SSC for 30 minutes twice at room temperature and in 0.1X SSC twice at 57°C for 1 hour. The nytran was then air-dried, wrapped in cellophane, and placed on Kodak X-O-Mat film overnight. The film was then developed in an automated developer. This film was then digitized as on an Ambis Optical Digitizer. The results were expressed as area under the curve in mm$^2$. This data was corrected by the ratio of total rRNA in its lane as compared to that in the first lane. This then corrected for discrepancies in RNA loading. Digitization of rRNA is described in the previous section.
DNA Probe and Labeling

DNA probes, for the use in detecting specific mRNA sequences, were obtained from the specified source as plasmids contained in the appropriate strain of E.Coli. The bacteria were propagated in LB media containing either ampicillin or tetracycline depending on the selectable marker. The cells were grown in a shaker bath overnight at 37°C. Plasmids were harvested using the alkali lysis method as described in Manitatis. Plasmids were purified by extracting one time with phenol and twice with chloroform. Inserts were then removed by restriction digestion and then isolated and recovered from agarose electrophoresis.
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