The role of the rat cytomegalovirus R78 gene in virus assembly

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List of abbreviations

AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
bp	Basepair
BSA	Bovine serum albumin
CaCl	Calcium-chloride
COS-7	Fibroblast cell line
CsCl	Cesiumchloride
DEAE	Diethylaminoethanol
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DTT	Dithiothreitol
E	Early
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetra-acetic acid
EGFP	Enhanced green fluorescent protein
EMEM	Eagle's Minimum Essential Medium
EtBr	Ethidiumbromide
FCS	Fetal calf serum
FDA	Food and Drug Administration
FITC	Fluorescein
gB	Glycoprotein B
GPCR	G protein coupled receptors
H_2O	Water
HAc	Glacial acid
HCMV	Human cytomegalovirus
HEK	Human embryonic kidney cells
IE	Immediately early
IMDM	Iscove's Modified Dulbecco's Medium
IPTG	isopropyl-beta-D-thiogalactopyranoside
KAc	Potassium acetate
kbp	Kilobasepair
L	Late
LB	Ligation buffer
LB	Loading buffer
LM-gel	Low melting point electrophoresis gel
MCMV	Mouse cytomegalovirus
NaCl	Sodium Cloride
NaOH	Sodium hydroxide
ORF	Open reading frame
ORI	Origin of replication
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pR78	Protein of the R78 gene
pR83	Protein of the R83 gene
R2MΦ	cell line of rat macrophages
Rat2	Cell line of rat fibroblasts
RCMV	Rat cytomegalovirus
REF	Rat embryo fybroblasts
RHEC	Rat heart endothelial cells
RNase	Ribonuclease
RPMI	RoswellPark Memorial Institute medium
SDS	Sodium dodecyl sulphate
TE	Tris EDTA buffer
Tris-HCl	Tris-hydrochloric acid
UL	Unique long
US	Unique short
UV	Ultraviolet light
wt	Wild type
X-GAL	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Preface

While writing this thesis for my graduation as a master in Clinical Molecular Life Sciences I gradually realised that this part of my life is coming to an end. After approximately four years behind books, it is time to graduate and put the theory into practice.

Of course I could not have finished my master and internship at the department of "Medical Microbiology" of the academic hospital Maastricht without the good help and support of some special people. I would like to say thanks to all of them.

First I would like to thank my promotor dr. Patrick Beisser for the guidance and support he gave me in the last six months. Thank you for the confidence and freedom in the lab, you showed me how to think scientifically. I am glad you gave me such an interesting project to work on. I would also like to thank dr. Frank Stassen for the interesting meetings on Friday. Thank you for your critical questions and for sharing your point of view. Next, my gratitude goes to Erik Beuken for all the help in the lab. Erik, you were always there for me when I needed help in the lab. You never complained about my numerous questions. Thanks for your patience. I would also like to thank the minor and major students that helped me with this project and Prof. Dr. Cathrien Bruggeman for giving me the opportunity to explore the lab.

Finally, I would like to say thanks to my family. Thank you for listening to my problems and for trying to understand what I was doing. Thank you for giving me the opportunity to study and to complete this master.

Thank you all for the support

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Abstract

The rat cytomegalovirus (RCMV) R78 gene is a very special gene. This is due to the fact that its sequence is close to that of a G protein-coupled receptor, and still the protein of this gene is not expressed in the membrane of infected cells. Previous research has proven that when this gene is modified, viral replication is less efficient than under normal circumstances, although DNA replication is not altered. The function of the R78 gene still remains unknown.

Looking at colocalization of the R78 gene product with other proteins might give new insights on the function of the R78 gene. For colocalization experiments two gene products were chosen, pR55 and pR83. The pR55 protein is a major envelope protein, also called glycoprotein B. This protein is responsible for the entry of the virion into the hostcells. The R83 gene encodes a protein that is part of the tegument proteins that are present between the capsid and the envelope.

During assembly of new virions, tegument is placed around the capsid and an envelope is formed. Using antibodies to colour pR78, pR55 and pR83, it is possible to see if pR78 colocalizes with one of these proteins. A rabbit anti-pR78 antibody was already available, but antibodies for the pR55 and pR83 were to be made. Making these antibodies was the main goal of this study.

Polyclonal antibody development using the electroporation technique requires correct DNA expression plasmids for the injection of the rats. These were made using standard DNA cloning techniques. These include DNA precipitations, ligations, transformations and transfections. Three different vectors were used in this study for the comparison of efficiency of antibody production. Several expression plasmids are now ready to be used for rat immunisation.

Samenvatting

Het rat cytomegalovirus (RCMV) R78 gen is een speciaal gen. Dit komt doordat de sequentie van dit gen sterke gelijkenis vertoond met de sequentie van G-eiwit gekoppelde receptoren. Toch komt het eiwit van dit gen niet tot expressie in het celmembraan van geïnfecteerde cellen. Onderzoek heeft uitgewezen dat modificaties in dit gen leiden tot een verminderde virale replicatie. Toch blijft the DNA replicatie in deze omstandigheden ongewijzigd. De functie van het R78 gen blijft tot op heden onbekend.

Door te kijken naar co-lokalisatie van het R78 gen product met andere proteïnes kan er meer informatie bekomen worden over de functie van het R78 gen. Twee proteïnes zijn gekozen voor deze co-lokalisatie studie, het pR55 and het pR83 eiwit. Het pR55 eiwit is een belangrijk enveloppe eiwit, dat ook wel glycoproteïne B wordt genoemd. Dit eiwit is verantwoordelijk voor het binnendringen van het virus in de gastheer cel. Het pR83 eiwit is een tegument eiwit dat zich tussen de capside and de enveloppe bevindt.

Tijdens de assemblage van nieuwe virussen wordt er tegument aangemaakt tussen de capside en de enveloppe. Door het gebruik van antilichamen voor het kleuren van pR78, pR55 en pR83 is het mogelijk om te kijken of het pR78 samen voorkomt met pR55 of pR83. Konijn antilichamen zijn beschikbaar voor het pR78, maar antilichamen voor het pR55 and het pR83 moesten aangemaakt worden. Het aanmaken van deze antilichamen was het hoofddoel van deze studie.

Polyclonale antilichaamproductie door het gebruik van de DNA electroporatie techniek kan alleen als de juiste DNA expressie plasmiden beschikbaar zijn voor de injectie van de ratten. Deze plasmiden werden gemaakt met standaard kloneringstechnieken. Deze omvatten o.a. precipitaties, ligaties, transformaties en transfecties. Drie verschillende vectoren werden gebruikt in deze studie voor de vergelijking van efficiëntie in antilichaam productie. Verschillende expressie plasmiden zijn nu klaar voor gebruik bij rat immunisatie.

1 Introduction

Human cytomegalovirus (HCMV) is a common pathogen that is part of the Herpesvirinae family. This is a family of large deoxyribonucleic acid (DNA) viruses that share some common features such as their virion morphology, their mode of replication and their capacity to establish latent and recurring infections. CMV is part of a subfamily of the herpesviruses, the Betaherpesvirinae. These can be distinguished from the other herpesviruses by several viral characteristics, like the genome structure, tissue tropism, site of the latent infection and their cytopathologic effect. Other examples of viruses from this subfamily are human herpesvirus 6.

HCMV infection occurs in approximately 2.5% of all newborns, and 50% of the adult population, with a prevalence that increases with age. After primary infection the virus will stay present in a latent form. This is a dormant form of infection, when there are no symptoms present. The virus will thus stay present in the host cells, for the rest of the host's live. Reactivation of the latent virus is possible and may occur at any time, but the risk for reactivation is much higher when the immune system activity is down regulated. The risk groups for reactivation are especially patients that undergo artificial immune suppression to prevent rejection of a transplant organ and people with a down regulated immune system like acquired immune deficiency syndrome (AIDS) patients [1].

Cytomegalovirus is acquired from blood, tissue and most body secretions, and causes productive infection in monocytes, macrophages and epithelial cells. Latency is established in T-cells and macrophages, but this can also occur in other cell types. Resolution of the primary infection can occur through cell-mediated immunity, but this immunity also contributes to the symptoms of the infection. CMV infections are asymptomatic in most cases, but when symptoms arise, these can be quite serious. Embryos, fetuses and immunocompromised patients are at high risk for symptom development. In these patients the virus can cause loads of damage [2, 3].

CMV is not highly infectious, and direct contact with contaminated material is necessary to get an infection. There are a number of different ways by which CMV can be transmitted. A mother can infect her unborn child transplacentally, because of a primary infection during pregnancy but also if the virus is already present in a latent form. Transmission is also possible at birth, when there are cervical or vaginal secretions containing virusparticles, and after birth, when the breast milk contains virusparticles. This is called perinatal infection. Other routes of virus transmission are through saliva, urine, tears, semen and cervical secretions. These are the transmission routes that mostly occur later in life [4].

When infection is already present at birth, 10% of the newborns show symptoms such as microcephaly, intracerebral calcification, hepatosplenomegaly and rash. Hearing loss and mental retardation are common consequences of this congenital infection. Infants born to mothers who went through primary infection during pregnancy are at higher risk for these manifestations than infants of mothers with a latent virus infection during pregnancy. Perinatal infection can also take place, but in healthy full-term infants this causes no symptoms. However when the infection is acquired through a blood transfusion, pneumonia and hepatitis can occur. In adults, CMV is mostly transmitted sexually. Most of these infections are asymptomatic, and only in immunocompromised patients, this causes disease. Pneumonia is a common outcome in these vulnerable patients and can be fatal if not treated. Other symptoms can be retinitis, intestinal pneumonia and encephalitis [2, 5, 6].
Antiviral treatments are already available for CMV patients, but these are not perfect yet. As soon as the treatment of the infection with antiviral products stops, reactivation of the virus is possible. Currently available agents, approved by the U.S. Food and Drug Administration (FDA) are Ganciclovir, Foscarnet and Cidofovir. These antiviral treatments are of real importance when it comes to immunodeficient patients. Treatment of these patients improves survival and quality of life. For immunocompetent infected patients with symptoms, these agents are only advised when there is a real need for it. When symptoms are not too serious, antiviral treatment is not used. This is because these agents are still toxic [7, 8].

1.1 Viral structure

Like all other viruses, CMV depends on the host cell for its life-sustaining functions and reproduction. To translate their viral DNA, CMV needs ribosomes from the host cell. The host cell is also a supplier of energy, amino acids, nucleotides and lipids for the virus. CMV viruses consist of a core containing a 235 kilobasepair (kbp) linear genome, composed of double stranded DNA [5, 9]. This DNA encodes the necessary information for the synthesis of all proteins needed for the survival and reproduction of the CMV, and is capable of encoding more than 200 potential protein products. Most of the protein functions however remain unknown or unclear [10].
The genome is packaged into a protein coat, also called a capsid. This is a rigid structure that is able to package and protect the DNA. The capsid has an icosahedral structure and consists of multiple protein subunits and it protects the DNA of the cytomegalovirus against digestion by enzymes. Together the DNA and the icosahedral capsid are called the nucleocapsid. In case of the cytomegalovirus, this nucleocapsid is surrounded by an

envelope composed of lipids, proteins and glycoproteins. This envelope is only maintained in aqueous solutions and is disrupted by drying, acidic conditions and detergents. When the envelope is disrupted, this leads to the inactivation of the virus, and so these enveloped viruses are transmitted in fluids. The structure of the envelope is quite similar to the structure of cellular membranes. The lipids form two layers around the capsid and the proteins intersperse these lipids. On this envelope, glycoprotein spikes are present. These are responsible for the interaction of the virus with the target cell. These glycoproteins are also the major antigens for the protective immunity.

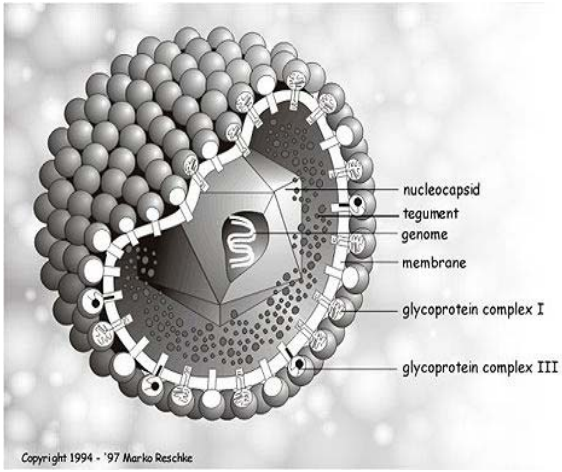

Figure 1. Structure of the CMV virion. The genome of the CMV virion, which is a double stranded DNA helix, is protected from enzymes by the capsid. Together the genome and the capsid are called the nucleocapsid. This nucleocapsid is surrounded by tegument proteins and the envelope membrane. On the envelope, several glycoproteins are present [11].

Between the nucleocapsid and the envelop there is an interstitial space, which is called the tegument. This interstitial space contains enzymes and proteins that help in the viral infection. When this viral structure is outside a cell, and thus when it can infect other cells, it is called a virion (see Figure1). One virion can contain multiple capsids with genomic DNA [12, 13].

1.2 Replication of CMV

All viruses use the same major steps for viral replication. They use their host cells for the delivery of the necessary substrates and energy for the synthesis of viral proteins and the replication of the genome. The replication of the CMV viruses in the infected cells occurs in three different temporal stages. During the early phase of infection, the virus recognises its target cell, binds to the cell and penetrates the plasma membrane. The genome is then delivered to the nucleus. After the early phase, the late phase begins.

This begins with the replication of the genome, and the syntheses of viral proteins. Viral assembly will then take place; this means that the nucleocapsid gets packaged into the tegument proteins, and the envelope. After this the virions are released. If the virus goes into a latent phase, there are no extracellular virions to be detected [13].
Each of these stages is characterised by specific genes that are expressed during these periods. There are three different phases of gene expression which are called immediately early (IE), early (E) and late (L). In the first hours after infection the immediately early genes get expressed. These genes encode non-structural proteins, like key regulatory proteins, which make sure that the virus is able to control the hostcell. The activation of these genes is controlled by the regulatory proteins of the host cell. The IE proteins also activate the genes of the early phase and they give a negative feedback to the IE genes. This means that the expression of IE genes will stop. The next phase of the replication can than begin [14, 15].
The early genes get expressed approximately twelve to twenty-four hours after infection of the cell. The proteins encoded by these early genes are responsible for the DNA replication, the formation of some structural proteins, but they are also responsible for the activation of the late genes. These late genes are responsible for the expression of structural proteins, and also for the assembly and maturation of the viral particles. When the assembly of new virions is complete, these virions are excreted and cell lysis will occur [5, 13].
The assembly of new virions occurs so that the genome is enclosed in a functional package. Assembly of the new virions occurs at different places in the host cell. DNA replication and packaging into the capsid occurs in the cell nucleus. After that, the nucleocapsid moves to the perinuclear space, right outside the nucleus, where the tegument proteins are added to the nucleocapsid. The envelope is created when the newly assembled viruses move out of the cell via a process that is called budding. These excreted virions can then infect other cells of the host, or they get excreted in saliva, urine or tears so they can infect other people [13, 16].

1.3 Viral genome

To study the characteristics and genome of cytomegalovirus, an in vivo model is required. However, CMV is a species specific virus, and thus an in vivo model is difficult to achieve for HCMV, which is a strictly human pathogen. Several animal models have thus been used to study CMV. Examples of these models are murine CMV (MCMV) and rat CMV (RCMV) [5].

Much work has been done to determine the sequence of the genome of CMV. Not only the sequence, but also open reading frames (ORFs) must be analysed. These ORFs are parts of the genome that could potentially code for a gene. When an ORF has a good size, it gives an indication for the presence of a gene. These ORFs, that are part of a gene, will always be in de translated region of the gene. The complete sequence of the rat cytomegalovirus of the Maastricht strain has been determined, and all ORFs have been analysed. The human CMV genome has also been analysed [17].
The human CMV genome contains 208 ORFs that are longer than 300 base pairs (bp). It consists of two segments, the unique long (UL) and the unique short (US). Both of these segments are flanked by inverted repeat regions. The organisation of other species specific CMV genomes is less complicated than that of HCMV. These genomes consist of a single unique region flanked by direct repeat regions. Loads of proteins, enzymes and cofactors are encoded by this DNA. Special attention however goes to the G-protein coupled receptors. Not much is known about these genes in viruses but some research has already been done [5].

1.3.1 G-protein coupled receptors

Of all genes found in the genome of the RCMV, there are two that show a sequence similarity with G protein coupled receptors (GPCRs). For RCMV these two are called the R33 and the R78 gene. G protein coupled receptors are essential for the signal transduction in cells. These receptors make sure that extracellular signals get to the inside of the cell by activating the right effector enzymes. GPCRs are encoded by prokaryotes and eukaryotes, but there are also some viruses, like the CMV, that encode these GPCRs. Gene variants of the R33 and the R78 genes are present in all species specific CMVs.
GPCRs are the largest superfamily of cell surface receptors. A common feature of these surface receptors is that they always have seven transmembrane helices. The protein pierces the membrane and in this way it is present in the cytoplasm, the membrane and in the extracellular space. The GPCR family is very important for pharmacology, because loads of the approved drugs use members of the GPCR family to reach their therapeutic effect [18].
The R33 and the R78 are the genes of the RCMV genome that show sequence homology to GPCRs. The R33 gene is very important for the pathogenesis of a RCMV infection. Using a null mutant that didn't contain the working R33 gene, Beisser et al. (1999) found that without the R33 gene, the RCMV was unable to infiltrate and replicate in the salivary gland epithelial cells of the infected rats [19].

1.3.2 The R78 gene

The R78 gene also codes a GPCR-like sequence. The sequence of this gene varies over different species. The R78 ORF shows 25% similarity with the MCMV ORF and 20% with the HCMV ORF. But the position of this gene in the genome of different species is comparable. The fact that the R78 gene is considered to be GPCR-like, depends on some major characteristics that this gene shares with GPCRs. First of all, there are seven predicted hydrophobic regions present in the R78 sequence. These are important for the localisation of the formed protein in the plasma membrane. There are also two cysteine residues present, which might be essential for the proper folding of the protein, and the last characteristic that this sequence shares with GPCR sequences is that part of this protein resembles the GPCR protein part that is responsible for the coupling of a G-protein. The R78 gene product has got a lot of the characteristics of a GPCR, but they show little similarity with any of the other GPCRs already known. Characterisation of this unique family of GPCRs can be very important for the development of new antiviral therapeutics.

For proper research in this area, mutant RCMV strains where made. In these strains, the R78 gene was partially or completely removed, and these where respectively called RCMVΔR78c and RCMVΔR78a. It was shown that interference of the R78 gene affected the replication of the virus in the infected cells. Also a lower mortality was observed in the infected rats. This lower mortality was observed both in the rats that where infected with RCMVΔR78a and RCMVΔR78c compared to rats that were infected with the wild type (wt) RCMV. Researchers concluded that the RCMV R78 gene plays an important role in replication and pathogenesis of the virus infection [19].

More detailed studies were done using a special recombinant RCMV strain, which expressed a modified version of the R78 protein (pR78). This strain makes it possible to look at the expression pattern of pR78 because there is an enhanced green fluorescent protein (EGFP) that is coupled to the R78 protein. This strain is called the RCMVR78G strain. Virustiters produced by this recombinant strain are 3 to 4 times lower than the virustiters produced by the wt virus strain. This is more than the virustiter of the RCMVΔR78a strain, which produces virustiters that are 50 times lower than those of the wt strain. But still this means that the expression of pR78-EGFP only partly replaces the native R78 gene (Figure 2).

The R78 gene is a G protein-coupled receptor. This means that it would be expressed in the membrane of cells. Using the EGFP-coupled mutant RCMV strain, RCMVR78G, it was shown that this is not true. The protein of the R78 gene was detected all over the cytoplasm of the cells, and not in the cell membrane (Figure 2). Electron-microscope photographs show that the expression in the cytoplasm is limited to the space just outside the nucleus, the perinuclear space.

The viral replication was also studied, and compared to the viral DNA load produced in the infected cells. It was seen that the deletion strain, RCMVΔR78a had significantly lower virustiters. However, the mutant strains had a viral DNA load that was comparable to the DNA load of the wt viruses (Figure 2). Using this information, it was concluded that the R78 gene had no impact on the DNA replication of the virus. Other steps in the assembly of new viruses however can be affected by the deletion of this gene [20].

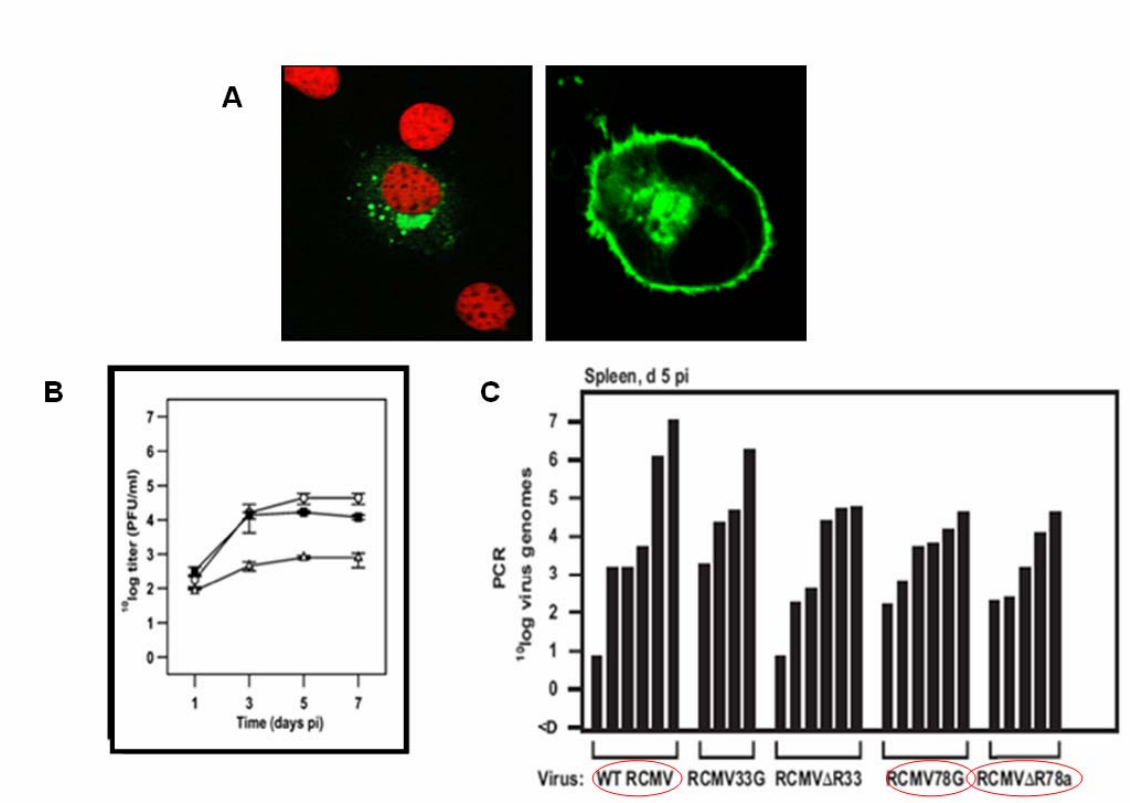

Figure 2: R78 characteristics. Kaptein et al. (2003) [20] performed some essential studies to get to know some characteristics of the R78 gene. (A) In the left photograph the R78 gene product is visible by the use of the EGFP tag (green). The nucleus was counterstained, and is seen in red. The gene product is seen in the cytoplasm, just outside the nucleus. The right picture shows the control, this is the BILF 1 gene of Epstein Barr virus (EBV) which is a G protein-coupled receptor, expressed in the cell membrane. (B) The viral replication of the wt RCMV (O), the EGFP tagged RCMVR78G (●) and the deletion mutant RCMVΔR78a (Δ) were compared, and a significant difference was seen. (C) Viral DNA load was measured in infected cells, using PCR. No significant difference was seen between the different mutant strains [19, 20].

1.3.3 The R55 and R83 genes

The R55 gene and the R83 gene are respectively important for the formation of envelope and tegument of the virus particle. These two genes are not G-protein coupled proteins like the R78 gene mentioned above. The R55 gene is part of a block of genes consisting of open reading frames encoding several proteins and polymerases. The protein encoded by R55 is also called glycoprotein B (gB). The primary structure of the translation product

of gB is a very good reserved structure. It is a component of the viral envelope, which occurs in almost all herpesviruses. The R55 gene has a vital function during the early phase of the viral infection, and during maturation [21, 22].

The protein of the R83 gene (pR83), also called phosphoprotein 65 in human CMV (pp65), is a major tegument protein. It is part of the late gene products that are made approximately 24 hours after infection. There are more than 25 different proteins present in the tegument, and they are all phosphorylated. pR83 seems to be one of the major phosphate acceptors in the infected cells [16]. Like almost all proteins present in the tegument, the R83 protein is also highly immunogenic.

These two genes, the R55 and the R83 are part of very important steps in the assembly of virions. This makes them good targets to study the assembly of the tegument, or the assembly of the envelope. Using antibodies to tract the location of their gene products, it is possible to compare their location to the location of a protein that is under investigation, in this case the R78 gene product. This can be done using colocalization studies using two different antibodies at the same time [16].

1.4 Aim of the study

The R78 gene is a very interesting gene: its sequence is close to the sequence of a GPCR, and still the protein of this gene is not expressed in the membrane of infected cells. When this gene is modified, viral replication is less efficient, although DNA replication is not altered. The function of the R78 gene is still unknown, and so we want to know something more about its function.

We want to do this by looking for colocalization of its gene product with other proteins. We want to see whether the R78 gene product colocalizes with the pR55 and the pR83 proteins in infected cells. The R55 gene is a major envelope gene, also called glycoprotein B, responsible for the entry of the virion into the hostcells. The other gene we are interested in is the R83 gene, which is part of the tegument proteins that are present between the capsid and the envelope.

During assembly of new virions, tegument is placed around the capsid and an envelope is formed. Using antibodies to stain pR78, pR55 and pR83, it is possible to see if pR78 colocalizes with one of these proteins. A rabbit anti-pR78 antibody is available, but antibodies for the pR55 and pR83 still have to be made. Making these antibodies is the main goal of this study.

Additionally we want to use a luciferase assay to study the activity of the viral R78 promoter. Streblow et al. (2007) [15] found that the expression profiles of RCMV genes are very different in vivo compared to in vitro patterns. The expression level is also much lower in vivo compared the in vitro expression levels. Differences in expression level are present between different tissue types in vivo, and in the paper they concluded that only 9 genes of the RCMV were expressed in all tissue types. One of these 9 genes is the R78 gene. The R78 gene appeared to be differentially expressed in all tissue types [15].

Given this information, we want to know when the promoter of the R78 gene is active. The luciferase assay will give information about the expression of the R78 gene. The expression can differ from expression at all times, to expression only at times of viral assembly or differential expression according to cell type and tissue type.

2 Materials and methods

2.1 Antibody development

Restrictions

For the creation of eukaryotic DNA expression plasmids the PubMed website [23], NEBcutter [24] and databases of the lab-department [25] were used. It had to be taken into account that all constructs had to stay in frame. Sticky and blunt ends were fused together using Klenow and deoxynucleotide triphosphates (dNTPs) on 5' overhangs.
For the BILF 1 vector, two kinds of inserts were used. First of all, parts of the R55 and R83 were used. The restriction sites used are summarized in Table 1. Oligo-sequences were also used as an insert (Table 2). The oligo sequences of the first 40-50 bps of the R55 and the R83 gene were ordered from SIGMA [26]. The vector itself was opened with the SmaI restriction enzyme.
For the pcDEF expression vector, which was opened with EcoRV, the whole gene sequence of the R55 and R83 genes was used (Table 2). The PIL excretion vector had to be opened with XhoI and NheI. For this vector, parts of the R55 and R83 gene sequences were used. Table 2 gives a summary of all restriction sites used. Restriction enzymes were inactivated by precipitation.

Ligation

The ligation of the inserts into the opened vectors was done using a ligation enzyme called ligase. The master mix used consisted of ligation buffer (LB), bovine serum albumin (BSA), adenosine triphosphate (ATP), dithiothreitol (DTT) and the ligase enzyme.

Transformation

Amplification of the correct expression plasmids is possible by transformation in competent bacteria like Escherichia coli (E. coli). When the expression plasmid DNA was added to these cells, a heat shock procedure was necessary so that the expression plasmids could enter the E. coli cells. All of these steps where performed on ice.
After the heat shock, the cells were incubated in medium to grow and then plated out on a bacterium plate containing a medium with the antibiotic to which the vector was

resistant. Overnight incubation at 37°C provided colonies on the plate, and it is then likely that these colonies consist of bacteria that have taken up the expression plasmid DNA, and expressed it.

Table 1: Overview of the used vectors and inserts. To make the right eukaryotic expression plasmid DNAs to produce antibodies, several inserts and vectors were used. Evaluation of the efficiency of antibody production with these vectors will be useful for future experiments.

VECTOR	INSERTS	RESTRICTION ENZYMES
BILF I membrane expression vector opened with SmaI	Parts of the R55 gene	
	Oligo-sequence of the R55	
	Parts of the R83 gene	• NcoI and PvuII (375bp) • PvuII and HincII (996bp) • HincII and EcoO109I (408bp)
	Oligo-sequence of the R83	
PcDEF expression vector opened with EcoRV	The R55 gene	• SexAI and NcoI (2859)
	The R83 gene	• NcoI (2103bp)
PIL excretion vector opened with XhoI and NheI	Parts of the R55 gene	• ClaI and EcoRV (899bp)
	Parts of the R83 gene	• NcoI and PvuII (381bp) • AccI and EcoO109I (407 bp) • AccI and NcoI (600bp)

Table 2: oligo-sequences. The oligo-sequences of the R55 and R83 genes, used for the BILF 1 vector are shown in this table. These were ordered from SIGMA

GENE	OLIGO-SEQUENCE
R55	Forward: 5'-GTACCGGCGCCATGCGGACCGCCTGGCCGACGGCGAGGTGGT-3'
	Reverse: 5'-ACCACCTCGCCGTCGGCCAGGCGGTCCGCATGGCGCCG-3'
R83	Forward: 5'-GTACCATGGACCACACCGAGCTCATCAAGCCGAACGACCCGATGTCGAGCTCCCCG-3'
	Reverse: 5'-CGGGGAGCTCGACATCCGGGTCGTTCGGCTTGATGAGCTCGGTGTGGTCCATG-3

Screening

Using long grafting sticks some cultures were taken from the bacterium plate, and these were transferred to a tube containing some medium with the correct antibiotic. By putting the tubes into a shaking incubator at 37°C, the cultures were able to grow. When the medium was saturated, 30µl were taken for the screening. This was added to an Eppendorf tube containing 30µl of phenol/chloroform and the same amount of 3x loading buffer (LB). These samples of the cultures were used to load a 1% agarose gel. The empty vector was used as a control.

After running the gel electrophoresis a staining was done to make the DNA bands visible. This was done with ethidium bromide. Under ultraviolet light (UV) the fluorescence of the ethidium bromide, which is bound to the DNA, was visible and a photograph of the gel could be taken.
Using a specific restriction enzyme, chosen for each insert, the direction of the insert was checked.

DNA isolation

A small DNA isolation can be done with 1.5 ml of saturated culture medium. This was done, mainly by using three basic solutions. The first solution is a mixture of tris-hydrochloride (Tris-HCl) and ethylenediaminetetra-acetic acid (EDTA). The second solution is composed of sodium hydroxide (NaOH) and sodium dodecyl sulphate (SDS). The last solution we add for the isolation of the DNA is composed of potassium acetate (KAc), water (H_2O) and glacial acid (HAc). These three agents make it possible to separate the cellular debris and the proteins from the DNA by centrifugation. After this precipitation, a purification of the DNA was done using phenol/chloroform, isopropanol and ethanol. The final pellet was resolved in ribonuclease (RNase).
When more pure DNA is needed, 0.5 L of saturated medium is used. The same solutions as in the small DNA isolation were used to acquire the first pellet. After resolving this in RNase, cesiumchloride (CsCl) and ethidiumbromide (EtBr) were used for the ultracentrifugation of the DNA. Afterwards the DNA has to be purified from the EtBr using ethanol and TE. TE is a buffer containing Tris and EDTA. After purification, the nucleic acid can be measured using the nanodrop, and dilation to 1µg/µl is practical for further use. The DNA was then stored in the freezer.

Transfection

For the transfection, we used fibroblast (COS-7) cells and human embryonic kidney (HEK) cells that were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5 or 10% fetal calf serum (FCS) respectively.
To transfect the COS cells we used diethylaminoethanol (DEAE). For the HEK cells however, lipofectamine was used. The cells were then grown in 24-well plates in medium containing the expression plasmid DNA. After an overnight growing period, the cells were fixated using formol and phosphate buffered saline (PBS). To look at the transfection results, the confocal microscope was used, and for this purpose the cells were transferred to microscope slides. Pictures could than be taken.

2.2 Promoter study

PCR of the promoter DNA

Primers were developed to use in a polymerase chain reaction (PCR). The forward primer was 5'-TTAGAGATCTTCGACGACGAGCACGTGCTGGT-3' and the reverse primer was 5'-TTAGAAGCTTCGTGGCTCGCACTCTGGAGT-3'.
Using these primers in a PCR, the DNA of the promoter was cloned several times. The PCR product was then purified using a MSB Spin PCRapache kit and checked by gel electrophoresis. The DNA was then cloned into a p-GEM-T-easy vector.

Control of the promoter DNA

After transformation a bleu and white staining was done using 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-GAL) and isopropyl-beta-D-thiogalactopyranoside (IPTG). DNA was isolated from the white cultures containing the promoter DNA. Using the enzymes Bgl II and Hind III, whose restriction-sites were added to the primers for the PCR, the inserted promoter DNA was restricted out of the vector. An agarose gel electrophoresis showed the length of the insertion DNA, which had to be 1 kb.
Using a PCR to get DNA, point mutations can occur, and so to be sure that the DNA of the promoter was unchanged, sequencing was necessary.

Cell culture

Different cell types were used for the luciferase assay. Rat macrophages (R2MΦ) were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FCS. Rat hart endothelial cells (RHEC) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 15% FCS. Rat fibroblasts (Rat2) were cultured in Eagle's Minimum Essential Medium (EMEM) with 10% FCS.

3 Results

3.1 Eukaryotic DNA expression plasmids

For colocalization experiments of the pR78 protein with pR55 and pR83, antibodies were needed against these proteins. Polyclonal antibodies can be made by immunisation of animals. Eukaryotic DNA expression plasmids were made for the immunisation of rats using the electroporation technique for a better delivery of these plasmids to the muscle cells of the rats. For the production of the DNA expression plasmids, several DNA cloning techniques were used.

3.1.1 Choice of the DNA Expression plasmids

DNA expression plasmids exist of a vector and an insert. The vectors used here are plasmids that contain an origin of replication (ORI) and a resistance gene. Their characteristics determine the expression pattern of the DNA expression plasmid. The inserts used are DNA sequences of the proteins against which antibodies are wanted, here the pR55 and pR83. To get the wanted DNA expression plasmids for the formation of antibodies, the used vectors needed to be treated with restriction endonucleases, and the inserts of the R55 and R83 needed to be cut out of their original vector. After ligation the vector and the insert form the correct DNA expression plasmid (Figure 3).

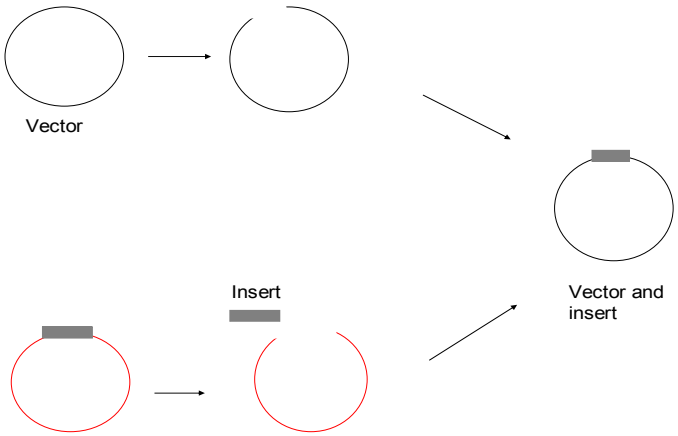

Figure 3: Ligation of the insert DNA into the wanted vector. The insert DNA has to be cut out of the original plasmid and isolated from the rest of the plasmid. The vector has to be cut open so that the vector and the insert can be cloned together to form the wanted DNA expression vector.

For the formation of the correct DNA constructs, three different vectors were used. Comparing their efficiency of antibody production may help to obtain antibodies more easily in the future. These three vectors were chosen for their differential expression pattern. The difference between these vectors is shown in Figure 4. For the BILF 1 vector, the insert is placed at the beginning of the BILF 1 gene. This was chosen because this part of the BILF 1 protein is expressed on the outside of the cell membrane (Figure 4). This BILF 1 gene is coupled to an EGFP, making it visible with fluorescence microscopy.
The pcDEF expression vector is used to express the R55 and R83 gene products in the cytoplasm. A promoter on this vector is responsible for their expression. For the PIL vector, the insert is positioned just behind a leader peptide sequence. Once expressed in the cell, the leader peptide is responsible for the relocation of the gene product to the outside of the cell. This leader peptide is internally linked to the cell membrane and this helps the protein to get through the membrane. Membrane proteases are then responsible for the separation of the two peptides.

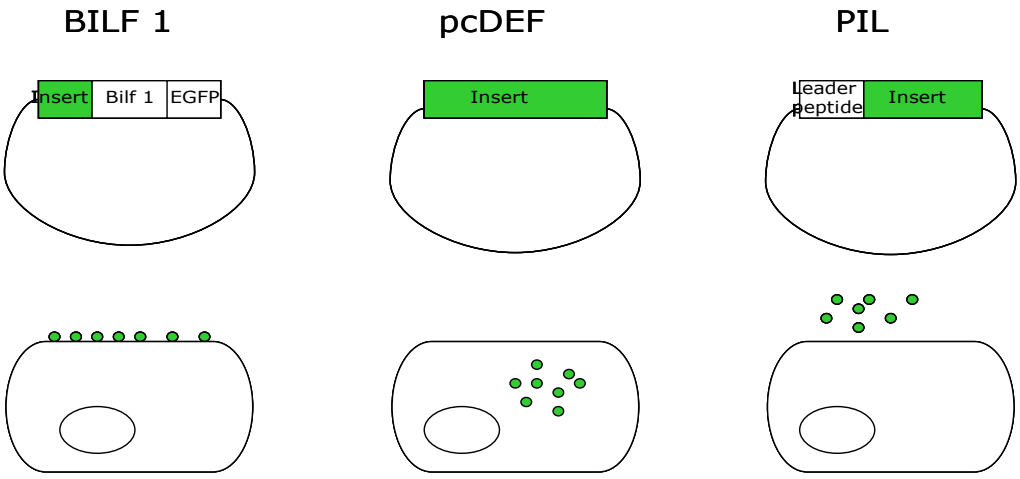

Figure 4. Different vectors used for the DNA constructs. Three different vectors were used for the formation of the DNA constructs. All three vectors are capable of expressing the inserted protein, but they all do this in a different way. The top figure explains the build-up of the vector, while the bottom figure represents the place in the infected cell where the construct protein gets expressed.

Different inserts were used in these three vectors. For the BILF 1 vector small inserts were chosen. This is because these inserts get expressed inside the BILF 1 protein. When the insert is too big, it possibly interferes with the protein formation. Protein formation is crucial for the membrane expression of the BILF 1 gene, and so interference with protein formation could lead to different expression patterns. To get two very small inserts for the BILF1 vector, oligo-sequences were ordered for the R55 and R83 gene. These oligo-sequences are approximately 40 bp big and represent the N-terminus of the gene.

The whole R55 and R83 genes were used as inserts for the pcDEF expression vector. This is possible because here the inserted gene is expressed without being part of a protein complex and there is no disturbance of protein formation. For the PIL excretion vector, which is a very big vector, parts of the R55 and R83 sequences were chosen. This makes cloning much easier and it also facilitates the excretion of the protein. In Figure 5 an overview is given of all the inserts, their length and the restriction enzymes used.

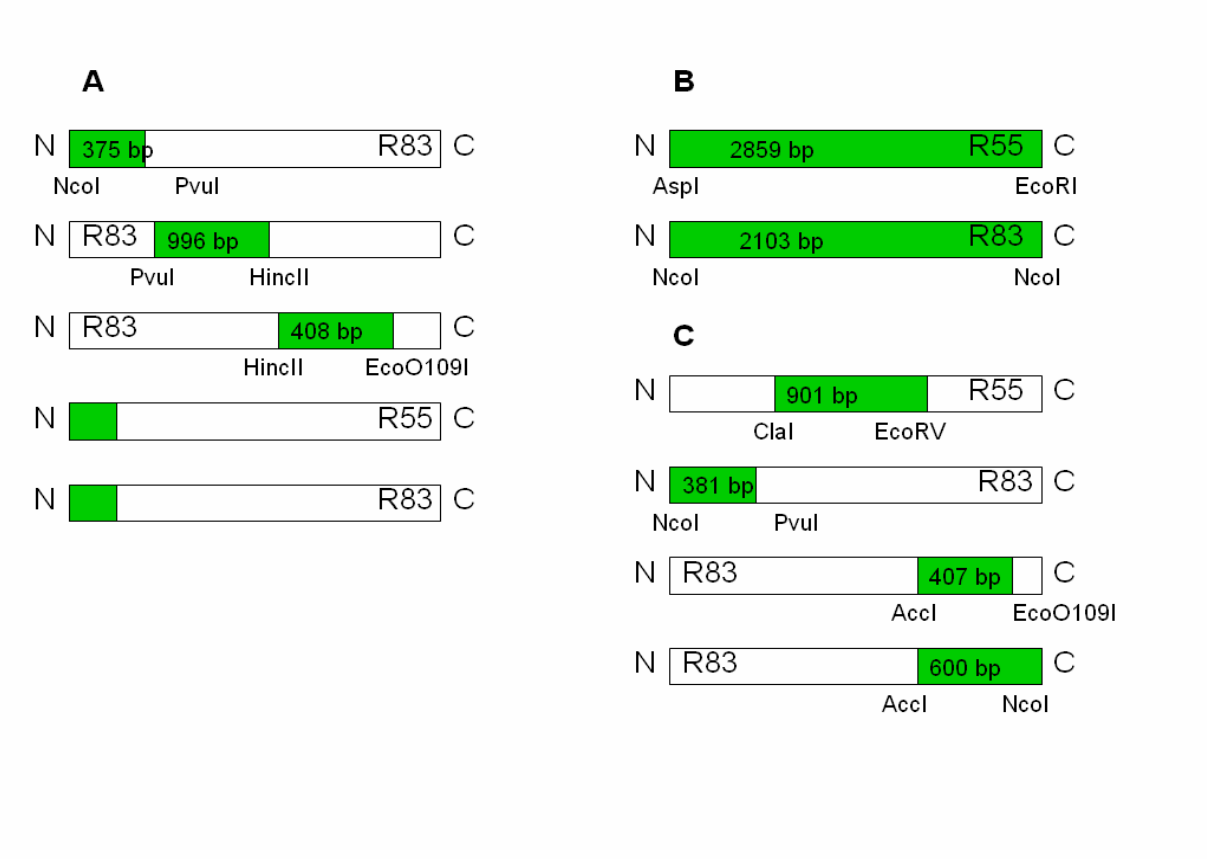

Figure 5: Overview of the inserts used for the DNA constructs. (A) The inserts for the BILF1 vector are small so that they don't disturb the protein formation. The two inserts at the bottom are oligo-sequences of approximately 40 bp. (B) The whole R55 and R83 genes were used as inserts for the pcDEF expression vector. (C) The inserts for the PIL vector were chosen to be small.

3.1.2 Construct formation

Vector and inserts were treated with restriction endonucleases and afterwards precipitated to inactivate the enzymes. A gel electrophoresis was done to control the length of the vector and inserts and to estimate the concentration of each sample. An example of such a gel is shown in Figure 6.

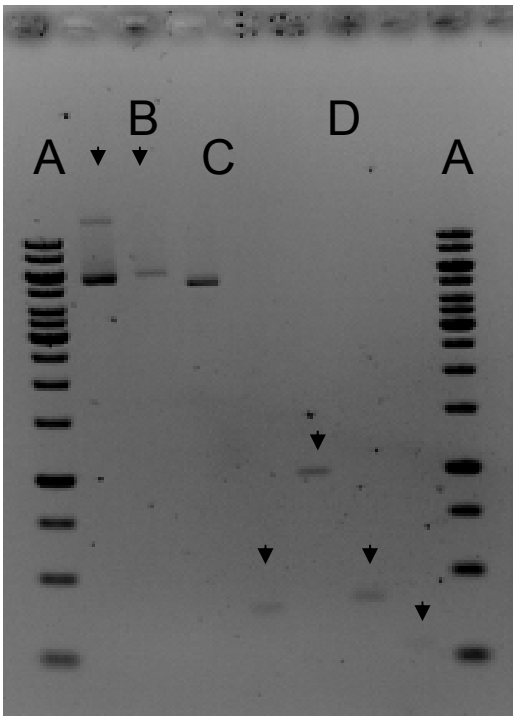

Figure 6: Gel electrophoresis of the DNA constructs. In this gel-picture we can control the restriction reactions and compare the DNA load of each sample. (A) A 1 kb DNA ladder is used to see if the inserts have the right length. (B) The unrestricted vector is use as a control for the restricted vector, but also the concentration of these 2 samples is known so other concentrations can be compared to these known samples. (C) The restricted vector should be higher up in the gel than the unrestricted vector, because of the difference in linearity. This vector is thus not restricted in the right way. (D) The 4 inserts present in this gel all have a different length. Comparison to the 1 kb ladder makes it possible to know whether these inserts have the correct length.

Together the vector and the insert form the correct DNA expression plasmid. However, the DNA needs to be checked after ligation to see if the ligation reaction has worked. A transformation of the DNA expression plasmid was done, and colonies were screened by gel electrophoresis. The empty vector was used as a control. Figure 7 is a gel photo of such a screening. When the right construct was found, the DNA had to be isolated to be used for transfection and antibody production.

The correct constructs were isolated using cesiumchloride and ethidium bromide to get rid of all toxins and to get very pure DNA. This isolation was done with 0.5l of saturated medium, so that in the end, we had DNA concentrations of proximately 1µg/µl. These concentrations can be used for transfection of cells, and for the animal studies.

All of the previous steps are can go wrong, and so the results are not always as expected. Often it is so that different steps in the protocol need to be adjusted before the expected result occurs.

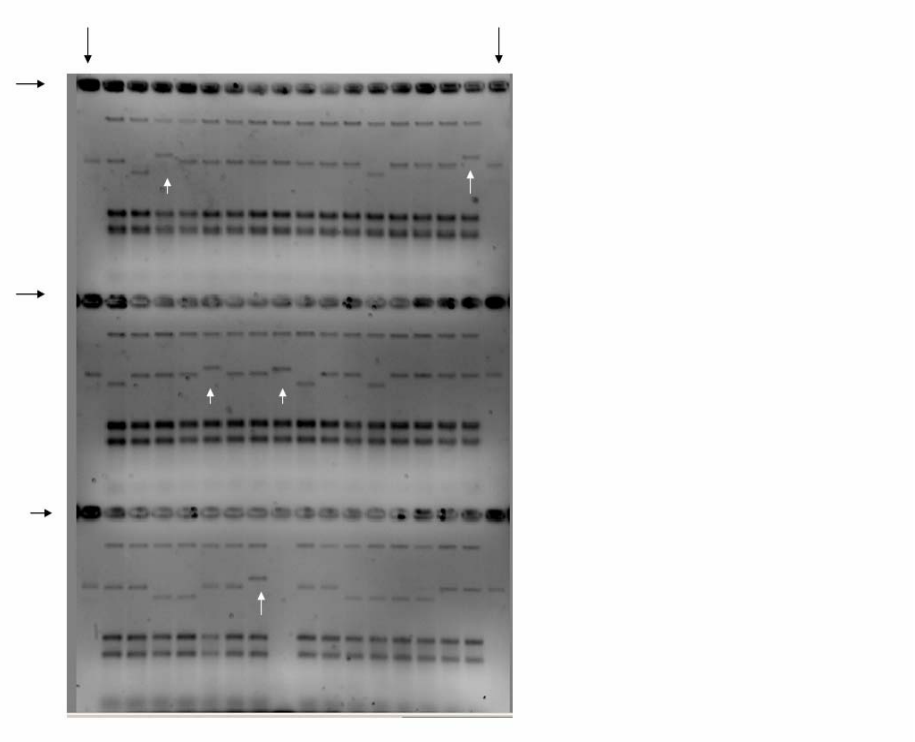

Figure 7: Gel picture of a fast screening. In this gel three slot-rows were filled with samples (see the arrows on the left). The control sample was loaded two times in each slot-row (see the arrows on top of the gel). The white arrows in the gel indicate the samples that contain a construct where the insert has become part of the vector. These samples are higher in the gel than the control sample.

3.1.3 Localisation of immunisation products

The correct DNA expression plasmids were used for a transfection. This means that the expression plasmid DNA was transferred to cells in culture. Here COS-7 cells and HEK cells were used. These were cultured in DMEM and than transfected with the expression plasmid DNA. Using this method, the expression plasmid DNA got expressed in the cells and because of the EGFP label in the BILF vector, the gene products could be seen by confocal microscopy. The expression pattern of the DNA expression plasmids in the cell is expected to be as shown in Figure 4.

In the PIL vector no EGFP label was present, and so an antibody colouring had to be done. The PIL vector contains a FLAG-tag. This is a protein tag that makes it possible to pinpoint the wanted protein using antibodies. The FLAG octapeptide can be detected by mouse anti-FLAG antibodies. A secondary antibody can be used to make the protein visible. Here we used anti-mouse fluorescein (FITC) labelled secondary antibodies. In the pcDEF vector there is neither EGFP nor a FLAG tag present. But for this vector the transfection is not required. In the two other vectors, a DNA fragment was cloned into a protein and so it is not sure that the protein will still be formed correctly. For the pcDEF vector this is not a problem, because here the whole gene is cloned into the expression vector and this gene is expressed without other proteins involved.

As seen in Figure 4, using these three vectors, it is expected to see three different expression patterns. When these expression patterns are present, this means that the DNA can be used for the electroporation protocol of the rats. Not all expression plasmids are already available and in table 3 an overview is given of the expression plasmids that are already formed.

Table 3: Finished and unfinished DNA expression plasmids. This table gives an overview of the expression plasmids needed for this project. The expression plasmids in black are already available, while the expression plasmids in red still need to be made.

VECTOR	INSERTS
BILF I membrane expression vector opened with SmaI	Parts of the R55 gene
	Oligo-sequence of the R55
	Parts of the R83 gene
	Oligo-sequence of the R83
pcDEF expression vector opened with EcoRV	The R55 gene
	The R83 gene
PIL excretion vector opened with XhoI and NheI	Parts of the R55 gene
	Parts of the R83 gene

For the BILF1 expression plasmids that are already available, transfections were done. First of all there are the BILF1 expression plasmids containing parts of the R83 gene. Three different expression plasmids were made using parts of the R83 gene as shown in Figure 3. All three expression plasmids were used for a transfection of COS-7 and HEK cells (Figure 8). The empty BILF1 vector was used as a positive control. This vector contains the gene of the BILF1-EGFP protein that is expressed in the membrane of transfected cells. Comparing the transfection results to the BILF1 control transfection it is seen that the DNA expression plasmids are expressed in the cytoplasm. For the p1014 and the p1017 expression plasmids expression is also seen in the cell membrane. The intensity of EGFP is comparable in for the DNA expression plasmids and the control BILF1 vector, which indicates that the expression of all plasmids is equally efficient.

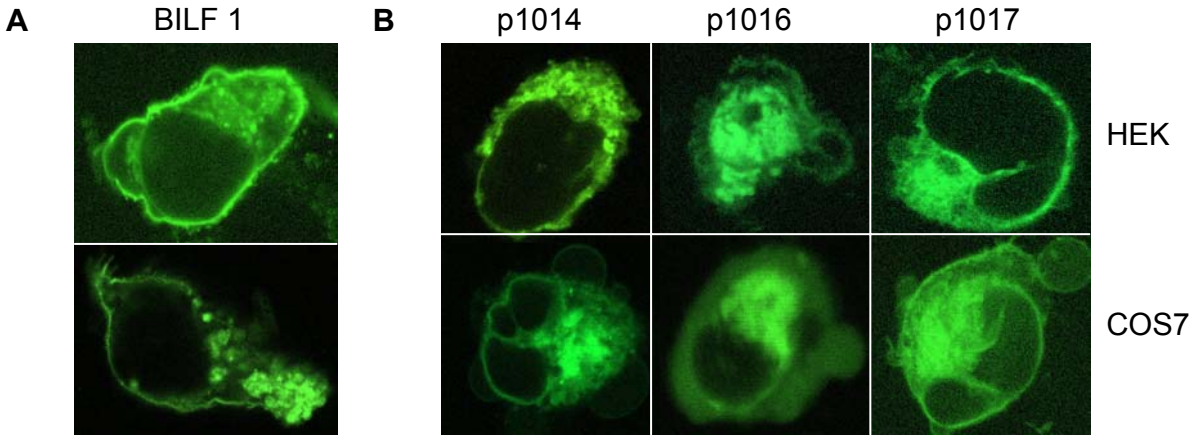

Figure 8: Transfection results of the R83 inserts. (A) The empty BILF1 vector was used as a positive control in HEK and COS-7 cells. (B) Three expression plasmids containing part of the R83 gene were used for the transfection. These were called p1014, p1016 and p1017 in the plasmid library of the lab, and respectively represent the first, middle and final part of the R83 gene that was inserted in the BILF1 vector as seen in figure 3.

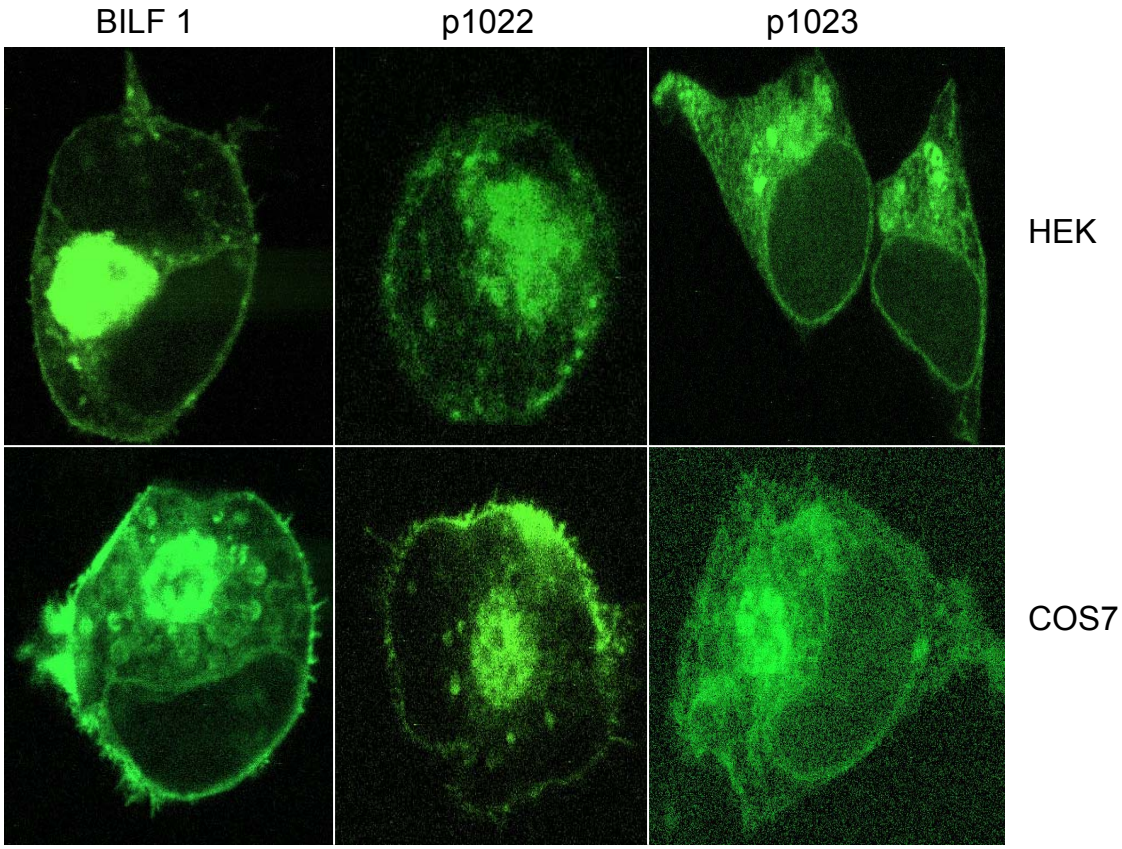

Figure 9: Transfection results of the oligo inserts. Pictures were taken with the confocal microscope. The empty BILF1 vector was used as a positive control for the two expression plasmids.

The expression plasmids of the BILF1 vector containing the oligo-sequences of the first nucleotides of the R55 and R83 gene were also used for a transfection of HEK and COS-7 cells (Figure 9). These plasmids were stored in the lab as p1022 for the R55 expression plasmid and p1023 for the R83 expression plasmid DNA. The intensity level of the EGFP marker is equal in all transfections, which means that the expression level of the expression plasmid DNA is the same for these oligo-plasmids. Expression of the DNA is seen in the cytoplasm and in the cell membrane. However, cell membrane expression is not as clear as in the control BILF1 vector.

3.2 Promoter study

Gene regulation of RCMV occurs in three different phases of gene expression which are called immediately early (IE), early (E) and late (L). In cultured cells these phases are tightly regulated. D.N. Streblow et al. used DNA microarrays to investigate the differences between in vitro and in vivo gene expression patterns, and also the differences of gene expression between tissue types.
In their paper they concluded that the expression profiles of RCMV genes are very different in vivo compared to in vitro. The expression level was also much lower in vivo compared the in vitro expression levels. They also studied the differences between different tissue types in vivo, and they concluded that only 9 genes of the RCMV were expressed in all tissue types. One of these 9 genes is the R78 gene. The R78 gene appeared to be differentially expressed in all these tissue types [15].
This article confirmed the importance of the R78 gene, and it suggested that the promoter of the R78 gene is constantly active. It is thus possible that this gene is expressed even if there are no viral proteins present. To look at the differential expression of the R78 gene a luciferase assay is an option. Therefore the promoter DNA has to be cloned into a luciferin vector.

3.2.1 Cloning the promoter DNA

Almost all viral gene promoters are located in a 1 kb region before the starting codon of the gene. To get this DNA, a PCR was done. Using primers designed to copy 1 kb before the R78 gene, the correct DNA could be cloned. These primers also contained a restriction site, to make cloning of this DNA simpler. Figure 10 gives an overview of the used primers.

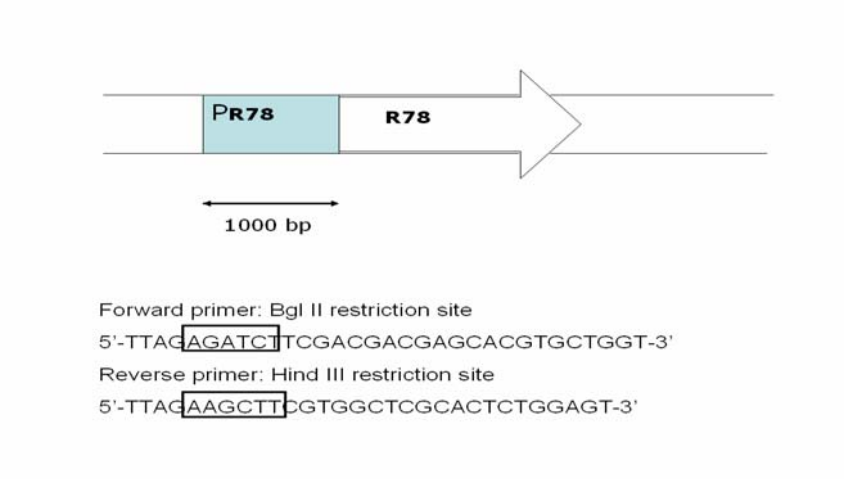

Figure 10: Promoter sequence. To get the promoter DNA a PCR was necessary and the primers used indicated the 1000 bp before the starting codon of the R78 gene. These primers contain a restriction site to make cloning of the promoter DNA much easier.

After the PCR reaction the DNA was checked and purified. A ligation was done to get the promoter DNA into the p-GEM-T-easy vector, which is a vector designed for sequencing reactions. A transformation was done using a blue and white staining. All white colonies were screened by gel electrophoresis. Figure 11 shows this screening.

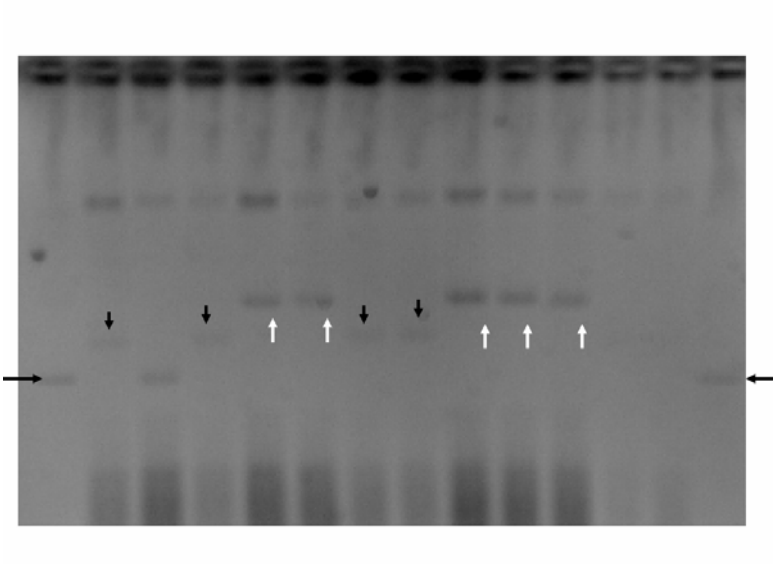

Figure 11: Gel electrophoresis of the promoter ligation into the p-GEM-T-easy vector. The arrows on the sides of the gel indicate the control. If a band is at the same height in the gel, there is no promoter DNA present. The white and black arrows in the gel indicate two different bands seen in this screening. Only one of these can contain the correct promoter DNA.

Two different DNA lengths are present in the screening, and only one can contain the correct promoter DNA. To know which expression plasmid DNA is the right one, part of the promoter DNA was cut out of the vector again using the restriction sites that were present in the PCR primers (Figure 10). Restriction with Bgl II and Hind III should cut out

the promoter DNA, being exactly 1 kb. This can be compared to a 1 kb DNA ladder in gel electrophoresis. By doing this it became clear that the middle length was the correct expression plasmid DNA, meaning the bands indicated by the black arrows.
There were four correct eukaryotic expression plasmids, meaning they had the correct length (Figure 11, black arrows). But during PCR mutations can take place, and so sequencing was necessary. Results showed that in all four plasmids mutations had taken place. Looking closely at the mutation chart of the second and the fourth expression plasmid with the correct size, a solution was found for this mutation problem (see Figure 12). The region between the BamHI and the NotI restriction sites is the only part of the second clone that contains a mutation. In the fourth clone, this region is free of mutations and so it is possible to replace the BamHI-NotI region of the second expression plasmid DNA by this region of the fourth plasmid. Using this technique a correct expression plasmid DNA could be formed.

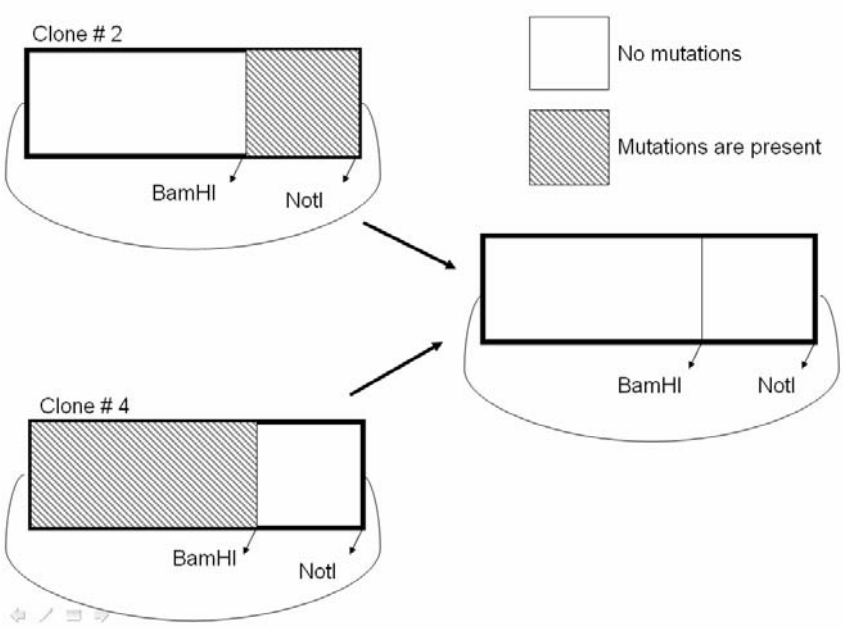

Figure 12: Solution for the mutation problem. Mutations in these two expression plasmids occurred at different places. By using the BamHI and the NotI restriction sites, it was possible to exchange the correct part of the promoter.

Both DNA expression plasmids were restricted using BamHI and NotI. For the second clone in figure 12 the p-GEM-T-easy vector with the promoter DNA was precipitated and the region between BamHI and NotI was lost. For the fourth clone in figure 12 the region between the restriction sites was isolated from a low melting point electrophoresis gel (LM-gel). A ligation of these two was completed and after transformation, a screening was done. The potentially good expression plasmids were than isolated and again sequencing was necessary to control the expression plasmid DNA for mutations. These promoter cloning experiments are still in progress.

3.2.2 Transfection optimalization

To do the luciferase assay, different cell types were needed to compare the promoter activity. All cell lines chosen are of rat origin because the R78 gene, which is of rat CMV origin, is under investigation. To start this luciferase assay when the promoter sequence is cloned into the luciferin-vector pGL3, the transfection of these cells needs to be optimal. So in advance, these cells were grown, and different transfections were done.
A macrophage cell line was chosen, called R2MΦ, and a fibroblast cell line, called Rat 2. The third cell line chosen for the transfections is called RHEC, this is a cell line of endothelial cells and for a positive control these cells can be infected with RCMV. Four different transfection techniques were used to see which one was optimal to use for the luciferase assay. The DEAE protocol and the lipofectamine protocol were already used for the transfections of HEK and COS-7 cells. The other two protocols are electroporation techniques for cell transfections, using the RF-modulator and the Biorad Gene Pulse Electroporator. For these test transfections, an EGFP vector was used. This makes it easy to look at the transfected cells and see whether the transfection has worked. The results of the RHEC cell transfections are shown in figure 13. The transfections of the R2MΦ cells and the Rat2 cells are shown in figure 14 and 15 respectively. For the Rat2 cells no pictures were taken of the biorad and RF-modulation electroporation. The results of the lipofectamine and DEAE protocol were sufficient in this case. Comparing the results of these different transfections, a protocol was chosen for each of these cell lines.

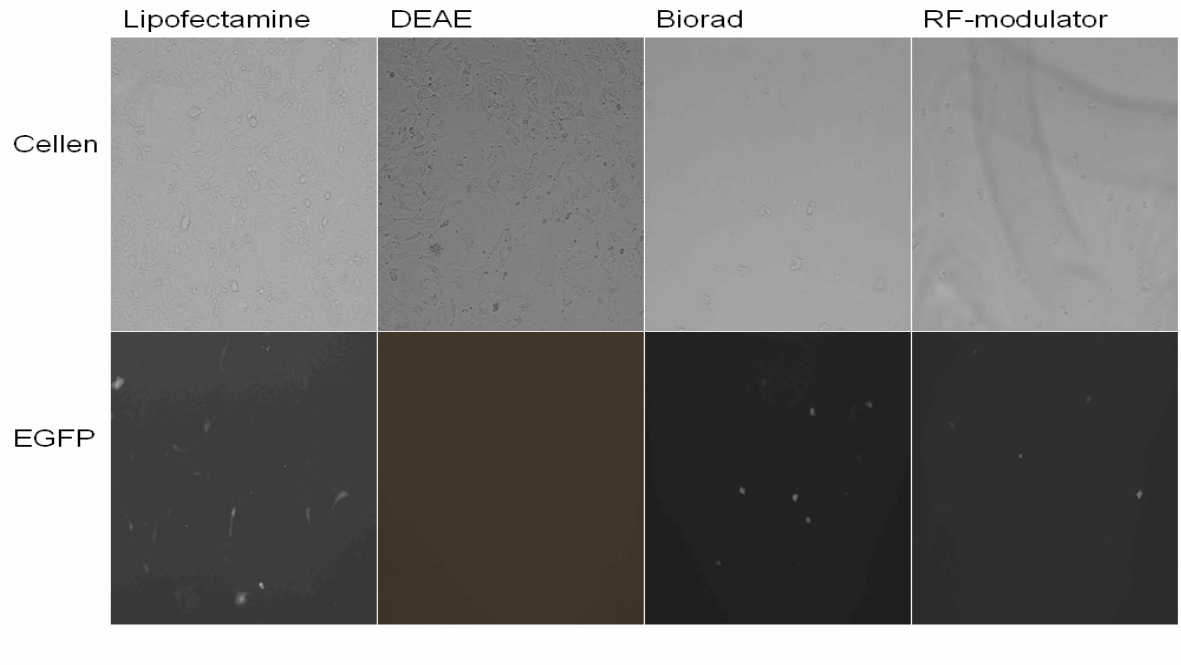

Figure 13: Transfections of the RHEC cells. Different transfection protocols were used for the transfection of RHEC endothelial cells. Comparing the total amount of cells to the amount of cells that is successfully transfected gives an indication of the best transfection method for these cells. The top pictures show the total amount of cells, the pictures at the bottom only show the cells that are successfully transfected.

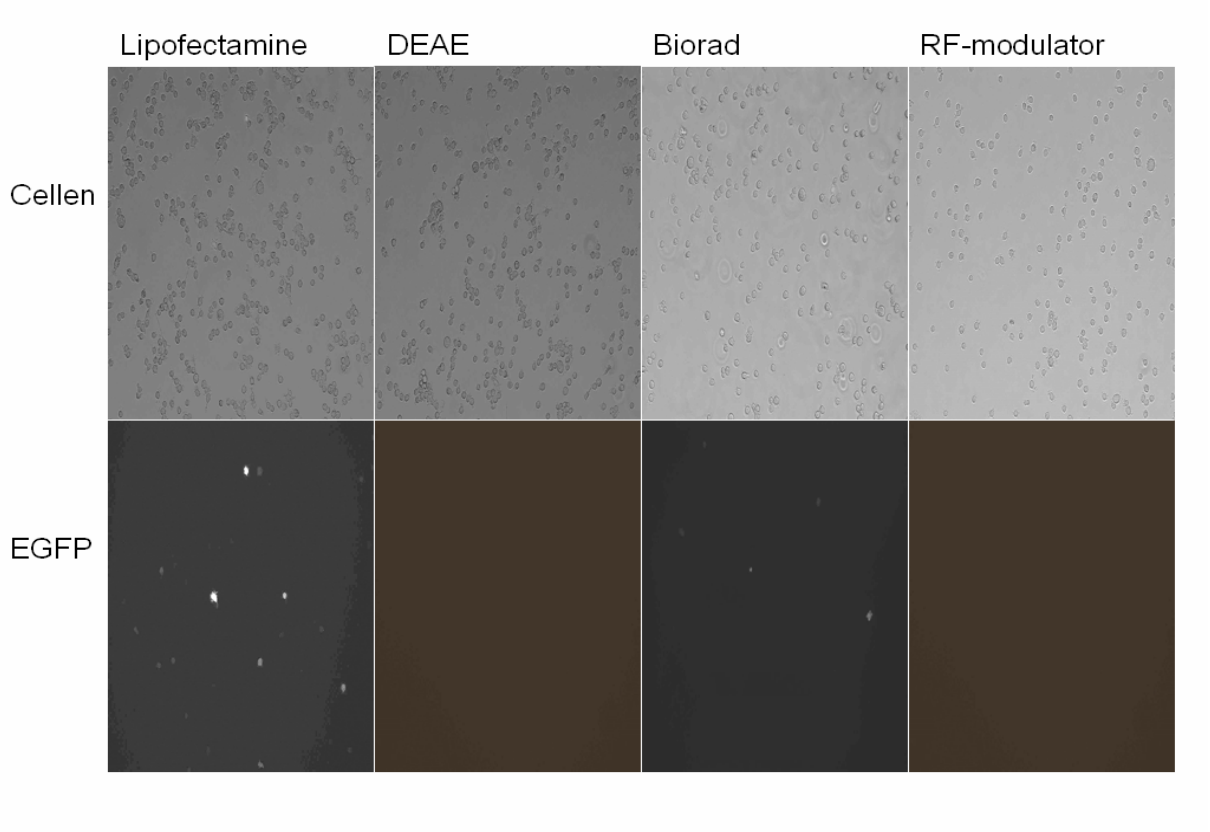

Figure 14: Transfection results of the R2MΦ cells. Different transfections are shown here for the macrophage cell line. In the top panel the total cell amount is shown. On the bottom only the cells that were successfully transfected are visible.

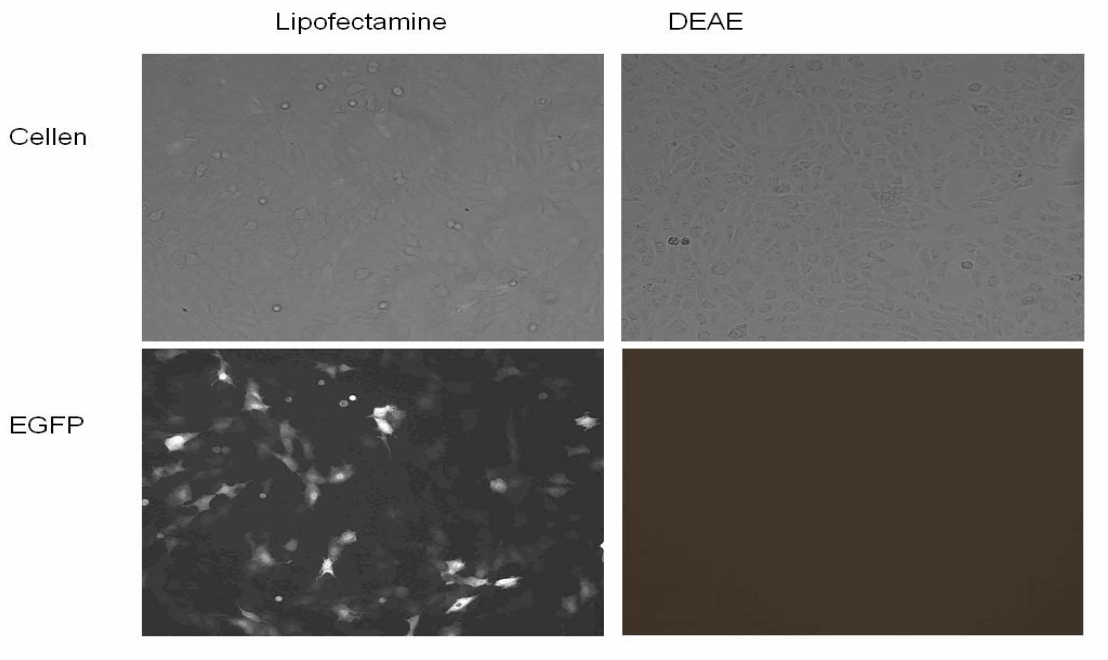

Figure 15: Transfection pictures of the Rat2 cells. The Rat2 cells were transfected using the lipofectamine and the DEAE protocol. On top the total amount of cells is shown. At the bottom only the cells that express the EGFP vector are visible.

To know which transfection protocol is best for each cell line, the amount of successfully transfected cells needs to be compared to the amount of total cells. In table 4 the percentage of cells that were successfully transfected in each protocol is shown.

Table 4: Successfully transfected cells. The amount of EGFP stained cells was compared to the amount of total cells for all transfection protocols. The results are given as percentages.

	Lipofectamine	DEAE	Biorad electroporator	RF-modulator
RHEC	7%	0%	23%	13%
R2MΦ cells	5%	0%	2%	0%
Rat2 cells	50%	0%	/	/

For the Rat2 cells it is clearly the lipofectamine protocol that needs to be used for transfections. For the RHEC cells the Biorad electroporator seems to be the best option and for the R2MΦ cells a choice needs to be made between the Biorad electroporator and the lipofectamine protocol.

4 Discussion

Getting to know the function of the R78 gene, and knowing when its promoter is active, will give new insights on CMV infection. The fact that the R78 gene encodes a G-protein coupled receptor-like protein might also be interesting for the development of new antiviral treatments against CMV. It is a fact that loads of antiviral treatments against different infections work through binding on a G-protein coupled receptor.
DNA expression plasmids were created to use for the immunisation of rats. However not all expression plasmids are ready yet (Table 3) and cloning is still in progress. For the DNA expression plasmids of the BILF 1 vector that are ready, a transfection was done (Figure 8 and 9). Here we could see whether the expression pattern was correct. Almost all constructs showed expression in the cell membrane as expected.
To see when the promoter of the R78 gene is active, a luciferase assay was chosen. The promoter sequence was obtained by PCR and further cloning is needed to get this sequence into the luciferin vector. An optimalization of cell transfections was done for the luciferase assay. Rat2, R2MΦ and RHEC cells were transfected in four different ways and for each cell type the best protocol was chosen. These cells were chosen because they are of rat origin and they represent different tissue types. Other options for the future are rat cell lines of different kinds of tissue, like rat embryo fibroblasts (REF).

DNA expression plasmids for antibody development

Transfection results show us that only p1016 was not seen in the cell membrane of the transfected cells (Figure 8). All other DNA expression plasmids that were used for transfection were seen in the cell membrane (Figure 8 and 9). The intensity of the EGFP-tags was comparable for all expression plasmids.
These data show us that all transfections have worked, however not all results are as expected. Looking closely at all transfection results, the transfections of the COS-7 cells succeeded more easily than the HEK transfections. This might be because these cells are better attached to the culture glass than the HEK cells. The COS-7 cell line is known in literature to be a good transfection host [27]. These cells show adherent growth to both glass and plastic surfaces. HEK cells are known to be adherent only when kept at 37°C for a couple of days. When these HEK cells are at room temperature there attachment to the culture flask disappears quite quickly[27]. This might have been the problem in our transfections. After fixation these cells are kept in a fridge and this might still interrupt their adherence.
For the p1016 DNA expression plasmid no cell membrane expression was seen. This could be due to the size of the insert used for this plasmid. The insert used here was

approximately 1 kb of length while the other inserts were smaller than 500 bp. The length of this insert might have interfered with the proper assembly of the BILF 1 protein, causing a problem for membrane localisation. Before proteins are relocated to their own place in the cell, and before they carry out their function, they need to assemble in the correct way. Membrane protein folding occurs though a complex process that occurs when the protein is coupled to the ribosome and the translocon [28, 29]. When amino-acids are added to a protein by cloning techniques, these amino-acids may have chemical features. They can be hydrophobic, hydrophilic or electrically charged for example. These amino-acids may interact with each other and with their surroundings and so they may influence the folding processes and interfere with cofactor binding [28]. This could have occurred in case of the p1016 plasmid. Here the gene product is not present in the cell membrane, which could be due to interference in the folding process of the seven transmembrane helices. Or the binding of this protein to the tanslocon could have been interrupted.

The p1014, p1017, p1022 and p1023 are ready to be used in the animal experiments, just like the pcDEF expression plasmid containing the whole R83 gene. P1014 and p1017 both contain an insert of the R83 gene and so only one of these expression plasmids will be used for immunisation. For the expression plasmids that expressed in the cell membrane, rats are already ordered and electroporation of these rats will start as soon as these rats are available. For the animal studies a protocol was written and approved by the ethical commission of animal use (Appendix 1).

When polyclonal antibodies are available their specificity can be tested using western blot techniques. These antibodies can then be used for the colocalization experiments with the R78 gene product. These colocalization tests can be done using fibroblasts and macrophages infected with wt RCMV or RCMVΔR78a.

Transfection optimalization for the luciferase assay

Different transfections were done to see which protocol was best for transfections for the luciferase assay. Results for our Rat2, R2MΦ and RHEC cells were very diverse as seen in Figure 13-15 and in Table 4. For all three cell types the DEAE protocol failed. This protocol has worked previously as seen in the transfections of COS-7 and HEK cells. Therefore it is possible that something has gone wrong during these transfections.

The data of these transfections show us how important it is to look for the optimal transfection protocol for each cell type. Lipofectamine treatment for transfections alters the plasma membrane and in this way it is possible for nucleic acids to cross the membrane and filtrate into the cytoplasm. Lipofectamine is a quite common transfection reagent, which has been used a lot in literature. DEAE is a cation that associates with the negatively charged DNA and carries it into the cell. However DEAE is toxic to cells and so

these transfections require careful optimalization [30]. This might be the reason why the DEAE protocol did not work in this experiment. DEAE transfections for COS-7 cells were already optimal from previous studies, but for the Rat 2, R2MΦ and RHEC cells no previous records are available in our lab.

Electroporation is also a way to induce DNA into a cell. Here an electric pulse temporally disrupts the cell membrane and in this way the DNA can enter the cell. Advantages of electroporation are its versatility and its efficiency [31, 32]. However cell damage can occur. In this study two different electroporators were used. For each of them a specific protocol was present; however this protocol was not cell type specific.

We can conclude that for the RHEC cells the protocol using the Biorad electroporator gives us the best results. For the other two cell types, R2MΦ and Rat2, the lipofectamine protocol was chosen. For the Rat2 cells this is an obvious choice, however for the R2MΦ cells both the lipofectamine protocol and the Biorad protocol were quite equally efficient. Here the lipofectamine protocol was chosen because in our lab we are more experienced and familiar with this transfection protocol.

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Appendices

Appendix 1

Begeleidingsformulier aanvraag dierproef DEC- UM [8] **Voorblad werkprotocol CPV [8]** *Versie 2006*	**DECNR[1#]:** **Ontvangen[#]:**

DEC datum goedkeuring#	Type aanvraag [2]
	Nieuw

VROM/GGONR[3]
GG)95-077 & IG04-081

LNV/CBDNR[4]

Hoofdproject	CARIM						

Deelproject	3						

Financieel beheerder	W. Mullers	Budgetnummer	30983120N

Titel van het onderzoek:

Detectie van virale eiwitten in geïnfecteerde cellen en weefsels

startdatum 1-4-2007 einddatum [9] 1-5-2007 *Duur van de proef [10]: 2 maanden*

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Diergroep	**1**	**.**	**.**	**.**	**.**	**.**	**.**
ctrl/exp/sham	exp						
Diersoort	rat						
Stam	Lewis						
Construct / mutatie ?	-						
Herkomst (leverancier) *	Harlan						
Aantal	32						
Geslacht	vrouwelijk						
Dieren immuuncompetent ?	ja						
Leeftijd/gewicht	11 weken						
Doel van de proef *	33						
Belang van de proef *	01						
Toxicologisch onderzoek *	01						
Bijzondere technieken *	14						
Anesthesie *	04						
Pijnbestrijding *	01						
Mate ongerief *	03						
Toestand dier einde exp*	01						

* VHI-coderingen zie bijlage

Verantwoording

Aanvraag dierproef DEC-UM **(kaders zijn licht flexibel, maar het geheel is max. 5 pag. versie 2006)**

Titel: Detectie van virale eiwitten in geïnfecteerde cellen en weefsels

1. Doel van de proef.

Binnen de capaciteitsgroep Medische Microbiologie wordt onderzoek gedaan naar verschillende betaherpesvirussen, zoals het cytomegalovirus (CMV) dat acute en levenslange infecties in zowel mensen als dieren induceert. Daarnaast wordt het Epstein-Barr virus (EBV) onderzocht, dat wordt geassocieerd met verscheidene lymfoproliferatieve ziekten, waaronder de ziekte van Hodgkin. Binnen de groep zijn verscheidene virale eiwitten gekarakteriseerd die mogelijk een cruciale rol spelen bij de pathogenese van CMV- en EBV-infectie. De precieze functies van deze eiwitten zijn nog niet bekend.

Om de functie van deze virale eiwitten te bestuderen is de volgende onderzoeksvraag geformuleerd: in welke fase van infectie en op welke plaats (in welke celsoort/weefsel) komen de eiwitten tot expressie? Het doel van de proef is om polyclonale antilichamen op te wekken in ratten tegen de virale eiwitten waarin wij geïnteresseerd zijn.

Om antilichamen op te wekken in ratten willen we gebruik maken van DNA-immunisatie, een techniek die reeds met succes werd toegepast in projecten 2002-29, 2003-28 en 2004-073 en in de literatuur beschreven is (o.a. Mir et al., 1999). Zes tot tien weken na DNA immunisatie worden de ratten opgeofferd om de sera te extraheren. Deze sera zullen worden gebruikt voor in vitro immuundetectie van de virale eiwitten.

2. Maatschappelijke relevantie en/of wetenschappelijk belang

Humaan CMV (HCMV) is 1 van de meest voorkomende virussen: ongeveer 50% van de bevolking ouder dan 35 jaar in Europa, Australië en Noord-Amerika is HCMV-seropositief. Hoewel HCMV -infectie in immuuncompetente individuen meestal zonder symptomen verloopt, kan infectie van immuungecompromitteerde personen levensbedreigende gevolgen hebben. Wij hebben duidelijke aanwijzingen dat bepaalde RCMV -genen een essentiële rol spelen in de pathogenese van infectie (Kaptein et al. 2003). Aangezien deze genen ook geconserveerd zijn in HCMV, is het waarschijnlijk dat deze HCMV-homologen een analoge rol spelen in infectie. De eiwitten die gecodeerd worden door deze HCMV -genen zijn dan ook zeer aantrekkelijke en belangrijke doelwitten voor nieuwe antivirale therapieën.

EBV is geassocieerd met verscheidene lymfoproliferatieve ziektes zoals infectieuze mononucleose (de ziekte van Pfeiffer), Burkitt lymfoma, en de ziekte van Hodgkin, maar ook met andere tumoren zoals nasofaryngeaal carcinoom (NPC). De mortaliteit bij patiënten met lymfoom-aandoeningen ligt rond 10%, bij NPC rond 50%. Het BILF1-genproduct is een potentieel doelwit voor nieuwe antivirale middelen.

3. Alternatieven

In het verleden hebben ‘klassieke’ methodes met behulp van recombinante eiwitten en synthetische peptides in zowel konijnen als kippen steeds gefaald om geschikte antilichamen gericht op te leveren. Het genereren van antilichamen tegen RCMV pR78 en verscheidene RCMV chemokine-eiwitten met behulp van de combinatie DNA-immunisatie/electroporatie is daarentegen succesvol verlopen. Gezien de slagingskans is deze methode de beste optie.

De DNA-immunisatiemethode is door Medische Microbiologie in samenwerking met Neurologie al succesvol toegepast voor het genereren van antilichamen tegen RCMV pR78. Om voor een vervolgstudie dubbele immuunkleuringen mogelijk te maken is het belangrijk om de antilichamen tegen de viruspartikel-

gerelateerde pR55- en pR83-eiwitten te genereren in ratten (voor gecombineerde rat- en konijn-antilichaamkleuringen).

Daarnaast zijn we zijn er in een vorig experiment niet in geslaagd om in vier konijnen antilichamen op te wekken tegen EBV pBILF1. Daarom willen we opnieuw pogen antilichamen tegen pBILF1 op te wekken, maar dan in ratten, omdat de methode in deze dieren bij Neurologie meer geoptimaliseerd is.

4. Ethische afweging

Het genereren van antilichamen tegen antigenen heeft altijd tot zeer bruikbare inzichten in biologische processen en bijbehorende pathogenese geleid.

De eventuele colocalisatie van RCMV pR78 met andere virion-eiwitten leidt tot bevestiging van de hypothese dat pR78 een rol speelt bij de assemblage van virus-partikels. Dit is een nieuw en onverwacht mechanisme dat waarschijnlijk zal gelden voor meerdere verwante herpesvirussen, zoals het humane CMV en de humane herpesvirussen 6 en 7, die allen een soortgelijk eiwit bezitten. Het leidt bovendien tot een nieuw paradigma binnen de farmacologie, waarbij voor het eerst wordt aangetoond dat GPCRs niet alleen een functie vervullen aan de buitenste membraan van een cel, waar ze signalen van buiten naar binnen transduceren, maar dat deze ook binnenin de cel interacties met andere eiwitten kunnen dirigeren. Omdat pR78 een GPCR is, kan n.a.v. de resultaten worden besloten een antagonist te ontwikkelen die pR78 en verwante virale GPCRs aangrijpen en daarmee betaherpesvirusreplicatie kunnen remmen.

De eventuele localisatie van EBV pBILF1 kan bepalen of het eiwit van belang is bij actieve replicatie. In dat geval kan worden besloten een antagonist te ontwikkelen die de replicatie zou kunnen remmen. Het eiwit zou ook van belang kunnen blijken te zijn bij latentie of reactievatie van het virus. Antagonisten zouden in dat geval kunnen zorgen dat het virus in een latente staat blijft onder omstandigheden waarbij het virus normaliter zou reactiveren. Dit 'in de kiem smoren' door remming van reactivatie is wellicht nog effectiever dan het remmen van een virus dat al aan het repliceren is. Remmen van reactivatie zou een uitstekende profylactische waarde kunnen hebben bij risicogroepen als transplantatiepatiënten. Remmen van replicatie zou een uitstekende therapeutische waarde kunnen hebben als aanvulling op de huidige antivirale middelen (synergistische werking).

Deze mogelijke nieuwe inzichten en het daaruit volgende besluit om antagonisten te gaan ontwikkelen wegen zwaarder dan het ongerief dat de ratten zullen hebben als gevolg van narcose, injectie, electroporatie en afname van bloedmonsters.

5. Wetenschappelijke onderbouwing

Cytomegalovirussen (CMVs) zijn species-specifieke betaherpesvirussen die acute en persistente infecties kunnen veroorzaken bij immuungecompromitteerde patiënten en neonaten. CMV gebruikt verschillende strategieën om antivirale verdedigingsmechanismen van de gastheer te omzeilen. Zo heeft het virus genen die homoloog zijn aan genen van de gastheer, waaronde chemokine-, cytokine- en G eitiwit-gekoppelde receptor (GPCR) -genen. Deze genen zijn vang belang om het virus te laten handhaven bij afweerreacties en maken het zelfs mogelijk het virus te laten verspreiden door het lichaam, mogelijk gebruikmakend van afweercellen als vehikel. GPCR-genen van het humane CMV zijn UL33, UL78, US27 en US28. In het rat CMV (RCMV) genoom vindt men ook genen die coderen voor GPCRs: (i) R33, een homoloog van UL33 en (ii) R78, een homoloog van UL78. Recente data tonen aan dat de eiwitten die gecodeerd worden door R33 en R78, i.e. pR33 en pR78, een cruciale rol spelen bij de pathogenese van RCMV-infectie (Kaptein et al., 2003). Zo is aangetoond dat de virale replicatie van een mutant RCMV, gemuteerd in het R33- of R78-gen, in vivo significant is afgenomen in vergelijking met wild-type RCMV. Gebaseerd op deze resultaten hebben wij als doel de functie van pR78 te onderzoeken. Voor pR78 zijn in een vorig experiment met DNA-immunisatie (projectnummer 2004-149) op succesvolle wijze polyclonale antilichamen in konijnen opgewekt. Het gebruik van deze antilichamen heeft verrassende resultaten opgeleverd. Expressie van pR78 geschiedt niet, zoals gebruikelijk is voor GPCRs, in de buitenste celmembraan, maar in een gebied dat geassocieerd is met de assemblage van viruspartikels (nog niet gepubliceerd). Om aan te tonen dat pR78 een rol speelt bij de virus-assemblage, moet worden aangetoond dat pR78 co-localiseert met viruspartikel-rerelateerde eiwitten. Voor co-localisatie-experimenten zijn er antilichamen nodig tegen verschillende varianten van de eiwitten pR55 (een virusenvelop-eiwit) en pR83 (een virusmatrix-eiwit). Het doel binnen deze studie is het daarom genereren van antilichamen tegen verschillende varianten van pR55 en pR83.

Het Epstein-Barrvirus (EBV)—ook lid van de herpesvirusfamilie—is een ethiologische factor voor Burkitt lymfoma en wordt geassocieerd met verscheidene lymfoproliferatieve ziekten. Recente data hebben aangetoond dat EBV ook codeert voor een GPCR-homoloog, pBILF1, die van belang is voor de infectie van het virus (Beisser et al., 2005). Aangezien de precieze functie van pBILF1 nog niet bekend is, willen we met deze studie meer inzicht krijgen in de rol die wordt vervuld door pBILF1. Aangezien kennis over waar en wanneer pBILF1 tot expressie komt, belangrijke aanwijzingen verschaft over hun functie, willen we met behulp van antilichamen de localisatie zowel op cellulair als op weefselniveau bestuderen. Het doel binnen deze studie is daarom het genereren van antilichamen tegen pBILF1.

6. Wetenschappelijke beoordeling

Dit project werd toegekend door de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO ZonMw projectnummer 901-02-224). Voor de beschreven proeven werd een vergunning verleend door het ministerie van Volksgezondheid, Ruimtelijke Ordening en Milieu (VROM), krachtens het Besluit Genetisch Gemodificeerde Organismen Wet Milieugevaarlijke Stoffen, onder nummer GGO95-077 en IG04-081.

5 Proefdier

7. Proefdier keuze

7.a. Soort / stam
Soort: rat
Stam: Lewis
Herkomst: Harlan

6.b. Sexe
Vrouwelijk. Het is beschreven dat immuunresponsen sterker zijn in vrouwelijke versus mannelijke dieren, met als gevolg dat de antilichaamproductie hoger is (o.a. Grossman et al., 1993), zie ook project 2004-073.

6.c. Aantallen
Het doel is het genereren van antilichamen maken tegen volgende virale eiwitten: EBV pBILF1, vier verschillende subunits van RCMV pR55 en vier verschillende subunits van RCMV pR83. Expressieplasmiden coderend voor elk van deze eiwitten zijn voorhanden. N.a.v. project 2006-043 is duidelijk geworden dat de antilichaamtiter bij 50% van geïmmuniseerde ratten boven de beoogde titel van 100 nmol l^{-1} zit (Martinez-Martinez et al., 2007, figuur 1). De kans dat de antilichaamtiter bij 4 ratten lager is dan de beoogde 100 nm l^{-1} bedraagt daarmee $0{,}5^4 \times 100 = 6{,}25\%$. Het slagingspercentage, de kans dat minstens 1 van de 4 ratten een effectieve antilichaamtiter heeft bedraagt hiermee 100–6,25 = 93,75%. Dit is een acceptabel percentage, waarmee wij voorstellen 4 ratten per 'antigeen' (expressieplasmide) te immuniseren.

Het is niet mogelijk meerdere expressieplasmiden te injecteren per rat, om het aantal dieren te reduceren, met één uitzondering: expressieplasmiden die het EBV BILF1-gen bevatten kunnen gecombineerd worden met een van de expressieplasmiden die het RCMV R55- of R83-gen bevatten.

Het aantal benodigde ratten bedraagt dientengevolge (4 x R55-subunits + 4 x R83-subunits) x 4 ratten per DNA-construct = 32 ratten. Bij 4 van deze 32 ratten zal het BILF1-construct bijgespoten worden.
Totaal: 32 ratten.

6 Dierproef

8. Experiment

DNA-immunisatie wordt steeds meer gebruikt als alternatief op 'klassieke' procedures om antilichamen in vivo op te wekken. De electroporatietechniek werd met succes toegepast in projecten 2002-29 en 2003-28 en blijkt zeer effectief en veilig te zijn.

DNA-immunisatie en electroporatie
Elk dier zal onder narcose worden geïnjecteerd (intramusculair, i.m.) met hyaluronidase (dit is een enzyme dat de permeabilizatie van de extracellulaire matrix verhoogt en zo DNA-diffusie vergemakkelijkt, zie o.a. Mennuni et al., 2002), 10 min later wordt een expressieplasmide (intramusculair) ingespoten en 5 minuten daarna wordt het dier geëlectroporeerd. Pre-immuun serum wordt vervolgens gecollecteerd (wanneer de dieren nog onder narcose zijn). Om uitdroging van de ogen te voorkomen, zal oogzalf worden gebruikt (Vitamine A oogzalf). Deze procedure zal worden uitgevoerd op dag 0 en daarna worden herhaald op dag 2 en dag 4. Deze herhalingen zijn effectiever gebleken voor de introductie van DNA in weefsel, zoals eerder beschreven in projectnummer 2006-043.

2-wekelijkse bloedafname
Om de antilichaamproductie te controleren over tijd, zal om de 2 weken vanaf de dag van DNA-immunisatie en electroporatie, bloed worden afgenomen en serum worden gecollecteerd. Aftappen van bloed zal gebeuren via een tailbleed. In vitro immunofluorescentie zal dan gebruikt worden om de aanwezigheid van antilichamen specifiek gericht tegen de targeteiwitten in het serum vast te stellen. Deze techniek zal tevens aangewend worden om te bepalen wanneer de dieren worden verbloed.

Collectie van totaal serum
Op het moment dat de in vitro resultaten uitwijzen dat specifiek antilichaam met voldoende hoge affiniteit aanwezig is, zullen de dieren worden opgeofferd. Onder narcose, zullen we de dieren verbloeden. We verwachten een voldoende hoge antilichaamtiter na 6-10 weken.

9. Experimentele condities

9a. Anesthesie
Voor electroporatie worden de dieren onder narcose gebracht met isofluraan. Ook zal er buprenorfine (0,1mg/kg) worden toegediend voor extra pijnstilling bij de ratten. Elk van de achterpoten worden geschoren en geïnjecteerd met 75 µl DNA oplossing in 0.9% NaCl (2 µg/µl). 5 Minuten na injectie wordt de poot met een geleidende gel tussen de elektrodes van het electroporatie systeem (Electro Square Porator ECM 830, BTX, San Diego, CA, USA) geplaatst. Er worden 8 pulsen van 200 V/cm, 20 ms, 1 Hz gegeven. Deze procedure wordt drie keer herhaald, telkens met tussenpozen van één dag (dus op dag 0, 2 en 4). Alvorens de dieren worden verbloed, worden ze onder narcose gebracht met isofluraan-inhalatie.

9b. Pijnbestrijding
Pijnbestrijding is niet nodig aangezien de dieren nagenoeg geen pijn zullen ondervinden gedurende de studie.

9c. Euthanasie en Humane eindpunten
Humane eindpunten: zoals hierboven vermeld, verwachten wij dat de dieren nagenoeg geen ongerief ten gevolge van de studie zullen ondervinden. Indien een dier binnen de onderzoeksperiode toch (onverwacht) duidelijk ziekte en ongemak vertoont (bijv. gedurende een paar dagen niet eten, significant gewichtverlies), zal het dier, in overleg met het CPV, worden uitgesloten van de studie en uit zijn lijden worden verlost met behulp van euthanasie (zie welzijnsevaluatie).

10a. Ongerief

De injectie van expressieplasmiden en de electroporatie vindt onder narcose plaats (zie punt 9 en 13; in totaal ongeveer 20 minuten), waarna het dier binnen een minuut ontwaakt. Deze handeling heeft na afloop geen negatieve effecten voor het welzijn van het dier.

Voor de 2-wekelijkse collectie van bloed, zal gebruik gemaakt worden van een tailbleed. Deze handeling gebeurt niet onder narcose, aangezien ze het dier slechts een matig, zeer kortdurend ongerief bezorgen. Ongerief-score = 03.

De verbloeding vindt plaats onder narcose en zal de dieren geen ongerief bezorgen.

10b. Welzijnsevaluatie

Het ongerief van de ratten wordt veroorzaakt door (i) narcose, (ii) lichte lokale inflammatie als gevolg van injectie en electroporatie (van dag 1 t/m 3, zoals ondervonden bij project 2006-043) en (iv) bloedafname. Het effect van electroporatie is minder ernstig dan het gebruik van adjuvans, welke bij klassieke immunisatie gebruikt wordt en bij DNA-immunisatie achterwege blijft. We verwachten dat hierdoor het ongerief bij de voorgesteld methode minder is dan bij de klassieke immunisatie. Desalniettemin zullen we het welzijn van de dieren in het algemeen en het inflammatieproces aan de poten blijven volgen door respectievelijk het wekelijks controleren van de gewichtstoename en dagelijkse inspectie van de achterpoten gedurende de eerste week.

11. Verzorging en huisvesting

De dieren worden in de ruimtes van het CPV gehuisvest. Zowel DNA-immunisatie en electroporatie als bloedafname zullen worden gedaan in de ruimtes van het CPV, met behulp van Mario Losen en personeel van het CPV.

12. Deskundigheid

Het gebruik van DNA-immunisatie om antilichamen op te wekken (zowel in ratten als konijnen), is al vaak beschreven (zie o.a. Mir et al., 1999; Chowdhury et al, 1998 en 2001; Mennuni et al., 2002). DNA-immunisatie in combinatie met electroporatie werd reeds veelvuldig met succes toegepast op ratten in de afdeling Neuropsychologie (DECnr 2002-29, 2003-28 en 2004-073). Voordeel van electroporatie is dat veelal slechts 1 injectie met DNA-plasmide (zonder injectie van adjuvant) nodig is (leidt tot stabiele expressie van het antigen). Onze studie zal in samenwerking met de afdeling Neuropsychologie gebeuren en de handelingen zullen worden uitgevoerd m.b.v. technisch opgeleid personeel van deze afdeling.

13. Standard Operation Procedures (SOP)

DNA-immunisatie en electroporatie
De ratten worden eerst in de inductie box geplaatst en daarna aan een kapje van het inhalatie anesthesie apparaat gelegd. Anesthesie inductie vindt plaats via een gasmengsel van lucht en isofluraan (4% voor inductie, 2.5% voor onderhoud). Buprenorfine wordt toegedient voor pijnstilling. Daarna worden de achterpoten geschoren en elk geïnjecteerd met 75 µl DNA oplossing (2 µg/µl) in 0.9% NaCl. Na 5 minuten wordt de poten met een geleidende gel tussen de elektroden geplaatst en worden er 8 pulsen van 200V/cm, 20 ms, 1 Hz gegeven (na vier pulsen worden de polen omgekeerd). De hele electroporatie procedure neemt ongeveer 10 minuten in beslag. Voor een hoge transfectie efficiëntie wordt de procedure drie keer herhaald, telkens met tussenpozen van één dag (dus b.v. op

dag 0, 2 en 4).

Collectie van bloedstalen
Bloedafname bij ratten zal 2-wekelijks gebeuren. Bloed wordt via een staartpunctie afgenomen. De ratten worden manueel gefixeerd in een handdoek. Er maximaal 150 µl bloed opgevangen in een heparine gecoat buisje. Alleen de laatste bloedmonsters worden post mortem via een hartpunctie genomen.

Relevante literatuur

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Annelies Vanheel

Datum: **15.06.2007**