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(54) Title: CORONA VIRUS–LIKE PARTICLES AS TOOLS FOR VACCINATION AND THERAPY

(57) Abstract

The present invention provides virus–like particles (VLPs) derived from corona viruses which are modified in various ways, genomically or in their protein composition, thereby exposing at their surface various biological or target molecules and/or carrying within the particles molecules with biological activity which need to be protected or shielded and/or containing genomes from which parts of authentic coronavirus genes or sequences have been removed or altered or into which foreign genes or sequences have been incorporated. The VLPs can for example be used as systems for the targeted delivery of therapeutic agents in the body or can be used as vaccine or as antigen in diagnostic tests.

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Title: Corona virus-like particles as tools for vaccination and therapy

The present invention relates to virus-like particles (VLPs) derived from corona viruses which are modified in various ways, genomically or in their protein composition, thereby exposing at their surface various biological or target molecules and/or carrying within the particles molecules with biological activity which need to be protected or shielded and/or containing genomes from which parts of authentic coronavirus genes or sequences have been removed or altered or into which foreign genes or sequences have been incorporated.

One of the major needs in present-day medicine is systems for the targeted delivery of therapeutic agents in the body. By consequence, the development of carriers that can direct cargo to specified groups of cells and introduce this cargo into these cells such that it can exert its biological activity, is a major challenge in biomedical research. Tremendous efforts have already been spent in the development and testing of systems based on liposomes, microspheres, antibodies etc. for delivery of drugs, genes, peptides and proteins. Though many of these approaches are promising, the actual successes so far are limited.
Viruses are transmissible agents which by their very nature carry out targeted delivery. In order to survive in the ecosystem they have shaped this feature to perfection. They have evolved structures and mechanisms to ensure that they precisely find the right target cells and introduce their genetic or enzymatic material into the cytoplasm without becoming trapped into the degradative lysosomal pathway.
By virtue of their combined targeting and delivery features, viruses intrinsically represent the most natural delivery systems and seem thus pre-eminently suitable as therapeutic carriers. Their exploitation requires that we can engineer virus-like particles and tailor them to their

new function. Such particles must be equipped with specific targeting information and "loaded" with a genetic or non-genetic message. The idea to use viruses for the better is not new but has been limited mainly to retroviruses and adenoviruses which may have limited value as tools for gene therapy.

Coronavirions have a rather simple structure. They consist of a nucleocapsid surrounded by a lipid membrane. The helical nucleocapsid is composed of the RNA genome packaged by one type of protein, the nucleocapsid protein N. The viral envelope generally contains 3 membrane proteins: the spike protein (S), the membrane protein (M) and the envelope protein (E). Some coronaviruses have a fourth protein in their membrane, the hemagglutinin-esterase protein (HE).

In infected cells the coronavirus nucleocapsids are assembled in the cytoplasm. The nucleocapsids interact with the viral envelope proteins which after their synthesis in the endoplasmic reticulum accumulate in the intermediate compartment, a membrane system localized between the endoplasmic reticulum (ER) and the Golgi complex. This membrane system acts as the budding compartment: the interaction of the nucleocapsids with the viral envelope proteins leads to the pinching off of virions which are then released from the cell by exocytosis.

We have recently demonstrated that the assembly of coronaviral particles does not require the involvement of nucleocapsids. Particles devoid of a nucleocapsid are assembled in cells when the viral envelope protein genes are co-expressed. The minimal requirements for the formation of virus-like particles (VLP's) are the M and E protein: the S protein is dispensable but is incorporated if present through its interactions with the M protein.

Biochemical and electron microscopical analysis revealed that the VLPs are homogeneous in size and have similar dimensions as authentic corona virions. Clearly, the M and E protein have the capacity to associate in the plane of cellular membranes and induce curvature leading to the budding of specific "vesicles" which are subsequently secreted from the cells. An article, incorporated herein by reference, describing these results has appeared in EMBO Journal (vol. 15, pp. 2020-2028, 1996).

In yet another article, corona virus like particles were shown which were not devoid of a nucleo-capsid, assembly here did not take place independent of a nucleocapsid (Bos et al., Virology 218, 52-60, 1996). Furthermore, packaging of RNA was not very efficient. Furthermore, neither of these two publications provides sufficient targeting and delivery features which would make the VLP's suitable as therapeutic carrier, for example being equipped with specific targeting information and/or with a genetic or non-genetic message.

The present invention provides coronaviruses and virus-like particles (VLPs) that are modified in various ways, genomically or in their protein composition, thereby exposing at their surface various biological or target molecules and/or carrying within the particles molecules with biological activity which need to be protected or shielded and/or containing genomes from which parts of authentic coronavirus genes or sequences have been removed or altered or into which foreign genes or sequences have been incorporated.
The S protein of these viruses is responsible for binding to the cell receptor and for subsequent fusion of viral and cellular membrane during entry. These two functions occur in separate regions of the molecule: receptor binding in the amino-terminal and fusion in the carboxy-terminal part. Therefore, by replacing (parts of) the receptor binding domain by biological molecules with different targeting

specificities coronaviruses can be directed to interact with a wide variety of target molecules that are for instance expressed on the surface of cells. Doing so without affecting the fusion function of the S-protein, the VLP according to the invention can fuse with or penetrate into cells not normally infectable by the original virus.

Provided by the invention are virus-like particles (VLPs) derived from coronaviruses in which one or more copies of the viral membrane proteins have been modified so as to contain foreign protein moieties of viral (either coronaviral or non-coronaviral) or non-viral origin, which moieties either are replacing part(s) of the VLP membrane proteins or are incorporated within these membrane proteins thereby constituting an integral part of them. By this the VLP is provided with novel biological properties such as new targeting means, or immunological information, or proteins with specific biological activity contained within the virus-like particle, which biological properties are associated with the VLP next to or in place of the natural spike protein of the original coronavirus.
In one embodiment of the invention, VLPs are provided in which (a part of) the ectodomain (i.e. the part exposed at the outside of the viral particle) of the spike protein has been replaced by the corresponding domain (or part thereof) of the spike protein of another corona virus. Hereby the VLP has acquired another cell substrate specificity whereby the VPL is capable of entering cells otherwise not accessible or susceptible to the original corona virus.
In a further embodiment of the invention, VLPs are provided which are composed of the mouse hepatitis coronavirus (MHV) M and E proteins and which contain chimaeric spike molecules consisting of the transmembrane + carboxy-terminal domain of MHV S but the ectodomain of the spike protein of feline infectious peritonitis coronavirus (FIPV). These VLPs can now enter feline cells and deliver MHV-like particles. Particles with these chimaeric spikes are produced by making constructs of the corona virus MHV S

gene in which the region encoding the amino-terminal domain is replaced by the corresponding domain of FIPV. These constructs are inserted into plasmids behind a bacteriophage T7 polymerase promoter. The constructs are then co-transfected with plasmids carrying the MHV M and E genes, both also behind the T7 promotor, in OST-7 cells which have been infected with a recombinant vaccina virus expressing the T7 polymerase. The resulting VLPs contain the chimaeric MHV/FIPV S protein. In another embodiment of the invention, the VLP is provided by the methods used as above with ectodomains of the spike protein of infectious bronchitis coronavirus (IBV), or the ectodomain (or part thereof) of an envelope protein of any enveloped virus not belonging to the coronaviruses. For example, MHV-based VLPs are provided by the invention which carry at their surface the ectodomain of the pseudorabies virus (PRV) glycoprotein gD instead of the MHV spike ectodomain or the luminal (i.e. amino-terminal) domain (or part thereof) of any nonviral type I membrane protein. In this way VLPs are provided that have a cell specificity for chicken cells, or pig cells, or cells reactive with the type I membrane protein.

In yet another embodiment of the invention, VLPs are produced with modifications that are contained within the particles. This is achieved by the incorporation of modified constructs of any of the corona viral proteins S, M, E and HE. In corona virus particles these proteins have their carboxy-terminal domain enclosed within the interior of the viral envelope. Thus, foreign protein sequences incorporated within, appended to or replacing the carboxy-terminal domain are enclosed as well. In this way, VLPs can be provided that contain protein moieties, or fragments thereof, from another virus, or non-viral proteins such as hormones, such as erytrhopoietin. This allows the production of VLPs containing a biological active protein or fragments thereof, which is/are shielded by the viral envelope and can be released and/or retrieved later, when the viral membrane is degraded or fused with another membrane. This allows the *in vitro* production in cells, or

the *in vivo* production in secretory glands such as milk glands of biologically active substance which are otherwise harmful or toxic to the producing cells, or which for other reasons need to be produced in a shielded form.

As another embodiment, MHV-based VLPs are provided carrying on their surface or inside an enzymatically active molecule like furin, or a cytokine, or a hormone receptor, or another viral or nonviral polypeptide with biological activity. In these examples, VLPs are provided with (additional) targeting means that serve to direct the VLP to cells otherwise not accessible to the original corona virus.

The invention provides VLPs which are modified at the ectodomain and/or the ectodomain of any of the viral proteins. By modifying the ectodomain of the spike protein, the VLPs are provided with modified biological molecules as targeting means that serve to direct the VLP to interact with other biological molecules that mirror or can interact with the target means, such as receptor proteins on cells, be it hormone receptors, specific immunoglobulines on B-cells, MHC and MHC associated molecules present on T-cells and other cells, transfer proteins or other receptor molecules known to the person skilled in the field of cell surface receptors. The targeting means can also be provided to interact with known binding sites of selected enzymes on proteins or other molecules that serve as substrate for the selected enzyme.

In a further embodiment of the invention, MHV-based VLPs are provided exposing an immunogenic determinant of a bacterial toxin. This is an example whereby the VLPs serve as immunogen or vaccine, here directed against the bacterial toxin. B-lymfocytes carrying the corresponding immunoglobuline at their surface are in this case the target cells for the VLPs, once recognozed by the B-lymfocyte, this cell(s) will multiply and produce the appropriate antibody.

Preparation of VLPs or coronaviruses with modified spikes can be achieved genetically by modification of the viral genome such that it expresses the modified S protein in infected cells. Here we also provide the preparation of coronaviruses containing altered spikes in a different way by expressing modified S genes in cells which are in addition infected with coronavirus. The co-incorporation of the mutant spike provides the virus with new targeting means. As an example we demonstrate the production of MHV particles containing the chimaeric MHV/FIPV S protein. The chimaeric S gene construct is expressed in L cells which are subsequently infected with wild-type MHV strain A59 (MHV-A59) or a mutant thereof. The progeny virus released by the cells contains the modified S protein. To demonstrate the altered targeting the virus was used to infect feline cells which are naturally not susceptible to MHV. The cells are now infected as shown by immunofluorescence and produce normal MHV.

As another example we demonstrate the production of MHV containing chimaeric S proteins in which part of the S ectodomain has been replaced by the corresponding part (i.e. the luminal or amino-terminal domain) of the human CD4 molecule, as an example of a nonviral protein. These modified coronaviruses have acquired the property to infect HIV-infected cells and cells expressing HIV envelope glycoprotein through the specific recognition of the CD4 and HIV gp120 complex. As a result, the HIV-infected cells will undergo a lytic infection, effectively reducing the number of HIV-infected cells in the body and thereby reducing the severity of the disease or even terminating the infection.

As another example, we demonstrate the production of MHV containing spike molecules of which the amino-terminal part has been replaced by a single chain-antibody fragment recognizing a specific cell surface protein that is expressed on cells that can normally not be infected with MHV. The modified virus is able to infect these otherwise refractory cells. This example illustrates the principle

that in this way, i.e. by inserting very specific targeting information into the viral spike, corona viruses can be directed to selected cells or tissues. The single chain-antibody fragment can for instance be selected in phage-display systems, or in other clonal selection systems of single-chain antibody fragments known in the field.
In the examples shown, the MHV was genetically unaffected. Consequently, the cells infected were eventually killed by the cytopathic effects of the virus. The modified viruses are thus powerfull tools to specifically kill predestined cells or tissues in the animal or human body. They are therefore suitable for therapeutic use, for instance for tumor therapy or for the killing of HIV-infected cells in AIDS patients.

Another aspect of the present invention relates to the use of said VLPs or coronaviruses as gene delivery vehicles. This can be achieved in different ways. One way is by incorporating foreign genes or sequences into the viral genome such that upon entry of the virus into cells these genes are expressed or that the inserted sequences become otherwise biologically active (as should be the case of ribozymes or antisense RNAs generated by the virus within the cells). The other way uses VLPs to package foreign RNA into particles by making use of the coronaviral packaging signal(s).
Incorporating foreign sequences into the coronaviral genome can be accomplished by genetic manipulation using an infectious (c-)DNA clone, a full-size DNA copy of the viral genome. It can also be achieved by RNA recombination in which case RNA representing part of the viral genome and containing the foreign sequences is introduced in infected cells allowing the foreign sequences to be incorporated through homologous recombination. Because coronaviruses will usually kill the cells they infect, it is important for most purposes to attenuate them so that they will not kill the cells with which they interact. Attenuation can be accoplished by genomically altering the virus through

deletion or mutation. Again, this can be achieved with a cDNA clone or by recombination.

As an example of the invention attenuation is provided by the preparation of an MHV mutant from which an essential gene has been deleted by recombination. A mouse cell line is provided in which the MHV E gene has been chromosomally integrated allowing the E protein to be produced by the expression of the gene. MHV lacking an E gene has been produced in normal mouse cells by recombination using a synthetic RNA containing a perfect copy of the MHV genomic 3'-end except for the lack of an intact E gene. The E-defective virus is able to grow only in the cells complementing the defect. The virus produced is attenuated such that it can infect other mouse cells, but non-productively: the lack of an E protein prevents the assembly of progeny.

As an example the principle of incorporating foreign genetic sequences into attenuated or not-attenuated VLPs or coronaviruses and of their expression is the following provided by the invention. An MHV derived VLP is provided into which a reporter gene such as LacZ or green fluorescent protein has been recombined and one in which the chimaeric MHV/FIPV S gene has been incorporated. The expression of the genes is shown by blue or green-fluorescent staining of VLP infected cells and by the acquired ability to infect feline cells, respectively.

The other way to obtain coronavirus-based delivery vehicles uses VLPs comprising foreign RNA sequences. Incorporation of foreign RNA sequences into these particles requires their packaging into nucleocapsids. Viral RNA-packaging by nucleocapsid (N) protein molecules occurs by the recognition of specific sequences, packaging signal(s) by the N protein. In MHV the packaging signal includes a 69 nucleotides long region in gene 1B. Foreign (noncoronaviral) RNAs containing the coronavirus packaging signal(s), or defective coronaviral genomes in which these signal(s) have been retained but into which foreign

sequences have been incorporated, are assembled into VLPs when introduced into cells expressing the N, M and E (±S) genes.

The VLP can introduce into a target cell a defined RNA that may have one of several functions. An example provided by the invention is a RNA acting as mRNA and specifying a particular protein such as a toxin or an inducer of apoptosis or an antibody fragment. Another example is an antisense RNA or an RNA with ribozyme activity. For most purposes it is essential to acquire multiple copies of the RNA in each cell to obtain the desired effect. This may not be feasible with VLPs which will only carry one or a few pseudo-NC. The invention thus provides the RNAs with amplification signals such that they will be multiplied in the target cell. To achieve this goal, Semliki Forest virus (SFV) replication sequences are used as the basis of the RNA construct. SFV-derived mRNA further comprising the coronavirus encapsidation sequences and specifying a reporter protein are assembled into VLPs. The SFV-driven amplification allows synthesis of the reporter protein in cells; in animals the appearance of antibodies to the reporter protein testifies to the productive delivery of the VLPs' content.

The invention also provides a VLP which is an antigen or epitope delivery vehicle meant for the induction of specific immune responses, cellular and/or humoral, systemic and/or local, including the induction and production of specific antibodies against proteins, to achieve protection against infection by pathogens, of viral and nonviral origin.

As an example the invention provides the induction of antibodies against the reporter protein derived from SFV-derived mRNA further comprising the coronavirus encapsidation sequences and specifying a reporter protein, as described above. As another example the induction of antibodies is demonstrated in mice to the FIPV spike and to PRV gD by immunization with the VLPs, also described above. Thus immune responses can be elicited both against proteins

which are encoded by the altered genome of the VLP and/or against proteins which have been incorporated as targeting means in the VLP, thereby partly or wholly replacing the original spike protein. The examples illustrate the applicability of the approach for the induction of immune responses against proteins as diverse as for instance viral, bacterial, parasitic, cellular and hormonal origins. As an example the induction of protective immunity in mice against PRV by the gD exposing VLPs is provided. The example illustrates the applicability of the approach in principle for vaccination against viral, bacterial and parasitic pathogens including for instance human coronaviruses.

The invention also provides VLPs for diagnostic purposes. In immunoassay always a great need exists for a well-defined, specific and sensitive antigen that can be prepared in large quantities. As an example the use of MHV-based VLPs carrying the PRV gD ectodomain in an ELISA to detect gD antibodies is provided.

The invention also provides VLPs which have fully maintained the original spike protein but which are altered genomically to attenuated the VLP and/or to encode nucleotide sequences that need to be delivered at the cells to which the original coronavirus was targeted. For example, in this way, intestinal epithelial cells, or respiratory epithelial cells, that are normally infected by TGEV, or PRCV, respectively, can now interact with VLPs derived from TGEV or PRCV, or other cell-specific coronaviruses if needed, to express proteins normally not expressed by said viruses. In this way, respiratory epithelial cells of cystic fibrosis patients can be induced to express lung surfactant molecules that are encoded by the altered genome of the VLP.

To further demonstrate the invention various examples are provided in the experimental part of this description which is not limiting the invention.

Experimental part

Coronaviruses are assembled intracellularly by budding into the intermediate compartment and, later in infection, into the endoplasmic reticulum (ER; Klumperman et al., 1994; Krijnse Locker et al., 1994). The cytoplasmically synthesized nucleocapsid (NC) - the viral genomic RNA packaged by nucleocapsid protein (N) molecules - interacts with cytoplasmically exposed domains of viral membrane proteins accumulated in the pre-Golgi membranes. Subsequent budding results in the formation of virions that follow the exocytic pathway out of the cell.

Recently, Vennema et al., 1996 discovered that the budding of coronavirus particles does not require a NC, whereas others (Bos et al., 1996) did not demonstrate the independance of VLP assembly from NC. The viral membrane proteins M, E and S, when synthesized by co-expression of their genes in cells, give rise to the production of coronavirus-like particles (VLPs) that are morphologically similar to normal virions (Vennema et al., 1996). Moreover, this process is not dependent on the spike (S) protein: spikeless particles were formed when only the genes encoding the membrane (M) protein and the envelope (E) protein were co-expressed. Incorporation of S into VLPs or virions is mediated by the M protein with which S associates (Opstelten et al., 1995).

The availability of the VLP system as provided by the invention provides us with an extremely valuable and convenient tool to study aspects of coronavirus assembly. This was demonstrated very clearly in a study of the structural requirements of the M protein for assembly. In this study we showed by co-expression of mutated M proteins with the E protein that particle formation is sensitive to changes in all domains of the M protein, i.e. the luminal N-terminal domain, the transmembrane domains and the cytoplasmic C-terminal domain. Particularly the identity of

the extreme C-terminus appeared to be very important; substitutions of the terminal residue can abolish VLP assembly nearly completely as does its deletion; deletion of the last two residues or more is fully fatal. To further demonstrate that the VLP assembly system is a faithful model for coronavirion assembly, we introduced several of these mutations into the viral genome by RNA recombination. The results were essentially fully concordant.

The aim of the further examples was to study the incorporation of the S protein into viral particles. In particular, we manipulated the ectodomain of the protein. The results again show that this domain can be replaced by that of an unrelated coronavirus; the interactions with the M protein that draw the S protein into a viral particle apparently reside in its transmembrane or cytoplasmic domain.

Materials and methods

Coexpression of genes; analysis of VLPs.

The mouse hepatitis virus strain A59 (MHV) M, E and S gene expression constructs, the procedures for the coexpression of viral genes in mammalian cells, the methods for the analyses of radiolabeled protein and VLP synthesis have all been previously described (Vennema et al, 1996 and references therein).

In addition to the polyclonal anti-MHV serum (K134), two monoclonal antibodies were used: J1.3 and A3.10 which recognize the ectodomains of the MHV M and S protein, respectively. Ascitis G73 was obtained from a cat infected with feline infectious peritonitis virus (FIPV) and contained antibodies to this virus' spike protein.

Construction of chimaeric S constructs.

Two expression constructs were prepared encoding mirror image chimaeric MHV/FIPV S proteins: one protein (designated S*) has the transmembrane + cytoplasmic domain

of MHV S and the luminal domain (i.e. ectodomain) of FIPV S, the other (designated S#) has the inverse composition. The construction made use of a convenient StyI site occurring at an identical position in both the MHV S gene and the FIPV S gene; this StyI site marks the location where in the S protein the ectodomain turns into the transmembrane domain.

The construction of pTFMS, encoding the S^* gene, used the pB1 cDNA clone (de Groot et al., 1989) as a source of FIPV strain 79-1146 S sequences. Using standard DNA manipultion methods a a chimaeric S gene construct was prepared consisting of the 3' StyI/BamHI fragment of the MHV S gene (Vennema et al., 1990) and the 5' coding sequence of the FIPV S gene spanning the AUG initiation codon down to the corresponding StyI site. The chimaeric gene was ligated as a BamHI fragment into the vector pTUG3 (Vennema et al., 1991) behind a bacteriophage T7 promoter.

The reverse chimaeric construct, encoding the $S^\#$ gene, was prepared from the complementary 5' BamHI/StyI MHV S gene fragment and the 3' StyI/SalI FIPV S gene fragment and was cloned into pTUC (Vennema et al., 1991). The plasmid was designated pMFS.

Cell fusion assay.

Spike proteins were expressed in subconfluent BHK-21 cell cultures grown in 3.5 cm dishes using the vaccinia virus system, now with strain Ankara (MVA) expressing the T7 polymerase (MVA-T7pol). Per dish 3mg plasmid DNA was transfected. From 2h p.i. cells were kept at 32°C. At 5h p.i. the transfection medium was taken off and indicator cells were added: *felis catus* whole foetus (fcwf-D), 5 x 10^5 cells per dish. The appearance of syncytia was monitored light microscopically.

Generation of recombinant MHV with chimaeric spikes

Homologous RNA recombination as descrived before (Fischer et al., [1997] and references therein) was used to

introduce the chimaeric S* protein into the MHV genome. In order to allow recombination of RNA at locations upstream of the S gene, the RNA donor construct pFV1 described by Fischer et al. (1997) was extended in the upstream direction of the S gene by incorporating 1.1kb sequences of the MHV gene 2. The chimaeric S gene was introduced into this plasmid as follows. Convenient restriction sites were first introduced into the modified donor construct at the 5' end of the S gene and just downstream of the 3' end of this gene. At the 5' end an AvrII site was engineered by mutating nucleotides 36 and 37 (both thymidines) in the S signal sequence encoding region into cytidines. Just downstream of the S gene the sequence TCTCCTGG was changed into the Sse8387I restriction enzyme recognition sequence CCTGCAGG. Subsequently, a chimaeric S* construct was prepared bordered by the same restriction sites. The AvrII site was engineered using the following PCR primer: 5'-CCTAGGGTATATTGGTGATTTTAGATGCATACAAGTTAACGTAACAC-3'. The Sse83871 site was created using the PCR primer TCTGTCTTTCCTGCAGGGGCTGTGAT. Finally, the chimaeric gene construct was retrieved using AvrII and Sse83871 and ligated into the donor plasmid that had been treated previously with the same enzymes. Capped RNA was transcribed from the resulting donor plasmid and used for electroporation into MHV-infected L2 cells to allow recombination (Fischer et al., 1997). The cells were plated onto a monolayer of FCWF cells to enable multiplication of the recombinant virus.

Results

Coexpression of protein genes and evaluation of antibody-specificities.

In order to biochemically demonstrate the expression of the MHV M, E and S protein and of the chimaeric S protein (S*) and to show their recognition by specific antibodies, the genes were (co)transfected into OST-7 cells infected with the recombinant vaccinia virus vTF7-3. The proteins were

labeled with ^{35}S-amino acids for 3h starting at 5h post infection (p.i.). Cells were then lysed, lysates were cleared by centrifugation and prepared for immunoprecipitation using various antibodies. The analysis of the precipitated proteins is shown in Fig.1. The M and S proteins were correctly expressed as shown by the immunoprecipitates obtained with the anti-MHV antiserum (K134) raised against purified MHV (lanes 1 and 3). The E protein (which requires a higher gel percentage to be resolved) is poorly recognized by this serum (not shown). The monoclonal antibody J 1.3 specifically precipitated the M protein (lanes 4 and 8), though with a lower efficiency than did K134. Another monoclonal antibody, A3.10, precipitated the S protein with high specificity and efficiency (lane 5). Finally, the antibodies in the feline ascitic fluid G73 recognized the chimaeric protein S* but not the MHV S protein (or the M protein; lanes 2,6 and 7).

Incorporation of chimaeric S protein into VLPs

To demonstrate the incorporation of the chimaeric S* protein into MHV VLPs two approaches were taken. In one approach we affinity-purified the VLPs from the culture media of the cells using the monoclonal antibodies and the ascitic fluid. The isolation of the particles was monitored by the appearance of the M protein as shown in Fig.2. Using the M-specific antibody J1.3 the release of VLPs was observed to occur when cells expressed M + E + S (lane 7) or M + E + S* (lane 1). Importantly, no M protein was picked up if the E protein was not present (lane 5), consistent with our earlier findings that VLP assembly minimally requires the combination of M and E (Vennema et al., 1996). The VLPs produced during coexpression of M, E and S could also be immunoisolated with the MHV S-specific antibody A3.10 (lane 8). This was, however, not the case when S had been replaced by S* (lane 2), as was to be expected since this monoclonal antibody recognizes the S1-domain of MHV S, which is lacking in the chimaeric protein. Conversely, the VLPs produced by the combination of M, E

and S* (lane 3), not those resulting from M + E + S coexpression (lane 6), could be immunoisolated by the G73 antibodies. That this result (lane 3) was indeed specific was additionally shown by the lack of M protein isolation by these feline antibodies after coexpression of only the M and S* proteins (lane 4). It should be noted that the direct demonstration of the S* protein in gels was was impaired by the strong copurification of an apparently secreted host cell protein with similar electrophoretic mobility.

To further prove the specificity of the affinity-purification approach, we also analyzed some aliquots of culture fluids in which the VLPs had been disrupted by the addition of detergent. Now no M protein was precipitated by the feline antibodies from the culture fluid of cells expressing the combination of M, E and S* (lane 9). This observation confirms that the precipitation of M seen in the absence of detergent (lane 3) is the result of the purification of S*-containing VLPs by the S*-specific antibodies.

In the other approach to demonstrate the assembly of the chimaeric S* protein into viral particles we pelleted the VLPs from the culture fluid of cells coexpressing the various genes. Cleared culture supernatants were layered onto a 25 % (w/w) sucrose solution and centrifuged. Pellets were dissolved and analyzed directly in gel. As shown in Fig.3 the production of VLPs was most easily seen by the appearance of the M protein in the pellets (lanes 1, 3 and 5). When no E protein was coexpressed, no M protein was observed as expected (lanes 2 and 4). Of the three S proteins coexpressed in this particular experiment it is clear that both MHV S (lane 1) and chimaeric S* (lane 3) are incorporated into VLPs. No incorporation was observed of the wild-type FIPV S protein (lane 5) nor of the inverse chimaeric S protein (S#, not shown).

Biological activity of chimaeric spike proteins.

In order to determine whether the chimaeric S* protein was biologically active, i.e. able to bind receptors on feline cells and to induce membrane fusion, a cocultivation assay was performed. The S* protein was expressed in BHK-21 cells. As controls the MHV S protein and the FIPV S protein were expressed in parallel as well as the inverse chimaeric spike protein S#, composed of an MHV-derived N-terminal (i.e. luminal) domain and a FIPV-derived transmembrane + cytoplasmic domain. Feline fcwf-D indicator cells were added 5h p.i. and the cultures were monitored for fusion. As illustrated by the light microscopical observations pictured in Fig.4, wild-type FIPV S protein but not wild-type MHV S protein induced syncytium formation as expected. Importantly, also the chimaeric S* protein was fully biologically active: extensive fusion was observed. This was not the case for the inverse construct, S#.

Generation of recombinant MHV with chimaeric spikes: Targeting of murine coronavirus to non-murine cells.

A derivative of the chimaeric S* gene was incorporated into MHV by homologous RNA recombination. Synthetic capped RNA transcribed from a donor DNA construct containing the S* gene was transfected into murine L2 cells that had been infected with MHV. The cells were plated onto monolayers of FCWF-D cells. The effect of recombination was evident from the formation of huge syncytia by the feline cells. No syncytia were formed when no RNA was transfected nor with any other donor RNA lacking the FIPV sequences. The recombinant virus harvested from the culture media was used to infect fresh FCWF-D cells.

Discussion

Noncovalent interactions with the viral M protein mediate the incorporation of spikes into the envelope of coronaviruses (Opstelten et al., 1995). In experiments described above we demonstrate that these interactions

occur in the carboxy-terminal domain of the S protein. The S* protein has only the 61 C-terminal residues of MHV S the remainder being derived from an unrelated coronavirus, FIPV. The results show that these 61 residues are sufficient to draw this chimaeric protein into viral particles. As this carboxy-terminal domain represents the transmembrane region and the cytoplasmic tail of the molecule, the ectodomains of the proteins are apparently not involved in interactions between the M and S proteins.

The above observations demonstrate the principle that foreign protein sequences can be incorporated into coronaviral particles. This was illustrated here by the incorporation of a spike ectodomain from an unrelated coronavirus but is feasible as well for other protein sequences from viral or nonviral origin. In addition, the principle is not limited to the spike ectodomain: modifications can be introduced also in other domains and/or other viral proteins provided that they do not compromise the potential to assemble viral particles.

The chimaeric S* protein was shown to have normal biological activities. Its ability to induce fusion of cells in a cocultivation assay demonstrated that the protein was transported to the plasma membrane of the BHK-21 cells in which it was expressed. In addition, it showed that the FIPV-derived ectomain was able to recognize the cognate receptor on the surface of feline cells and to functionally bind with it inducing the blending of the plasma membranes of the cells. These observations indicate that the S* protein is fully active. Consequently, when incorporated into MHV-based viral particles the protein targets the particles to feline cells and induce their fusion with these cells. This is also what we observed when the chimaeric protein gene was introduced into the MHV genome by recombination. The recombinant virus produced has acquired the ability to infect feline cells, which are normally not infectable by MHV. The finding provides

further evidence that it is possible to change the targeting properties of coronaviruses by modifying the spike protein, allowing the viruses to cross the species barrier.

LEGENDS TO FIGURES

Figure 1: Biochemical analysis of expressed viral proteins. OST-7 cells grown in parallel in 3.5cm culture dishes were infected with vTF7-3 and transfected after 1h with one or more of the plasmids pTUMM, pTUMS, pTM5ab and pTFMS (5mg per plasmid). They were incubated at 37°C. At 5h p.i., after a starvation period of 0.5h, they were radiolabeled with ^{35}S-amino acids (100 mCi/dish) for 3h after which the culture fluids were taken off and the cells washed with PBS containing Ca^{++} (0.9mM) and Mg^{++} (0.5mM) and solubilized in 600 ml/dish lysis buffer (20mM Tris-HCl [pH7.6], 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mg of aprotinin, leupeptin, and pepstatin). Lysates were cleared by centrifugation in an Eppendorf centrifuge for 10 min at 10,000 rpm. Immunoprecipitations were done using 200ml-aliquots of cleared lysate which were diluted with 800ml immunoprecipitation buffer (20mM Tris-HCl [pH7.6], 150mM NaCl, 5mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1mg of protease inhibitors per ml) before adding the antibodies (2ml K134, 3ml G73, 150ml J1.3 or 20ml A3.10). After overnight incubation at 4°C 30ml Pansorbin (Calbiochem) suspension was added and incubation continued for 1h. Immune complexes were then pelleted and washed 3 times with wash buffer I (20mM Tris-HCl [pH7.6], 150mM NaCl, 5mM EDTA, 0.1% Nonidet P-40) and once with wash buffer II (20mM Tris-HCl [pH7.6], 0.1% Nonidet P-40). Final pellets were suspended in Laemmli sample buffer, kept at room temperature for 10 min, heated at 95°C for 2 min, cleared by centrifugation at 10,000 rpm for 5 min and analyzed in 15% polyacrylamide gel. In the figure the combinations of plasmids that had been co-transfected and the antibodies used to immunoprecipitate proteins are indicated as are the positions of the M, S and S* protein.

Figure 2: Analysis of VLPs by affinity purification. The culture fluids obtained in an experiment as described for Fig.1 were cleared by centrifugation at 4,000 rpm for

10 min in an Eppendorf centrifuge. Antibodies were added to 600ml of cleared culture medium and immunoprecipitations were performed as described in the legend to Fig.1 except that the immune complexes were now washed 4 times with TEN-buffer (40mM Tris-HCl [pH7.6], 150mM NaCl, 1mM EDTA). In addition, to some 600ml-aliquots of cleared culture medium 150ml of 5 times concentrated lysis buffer was added before the antibodies were applied in order to solubilize VLPs, and immunoprecipitates were performed as described for Fig.1. The combinations of expressed proteins and the antibodies used are indicated at the top of the figure. Lanes 1-8 represent analyses of affinity-purified VLPs; lanes 9-12 are gel analyses of proteins precipitated after disrupting the VLPs by adding detergents to the culture fluid.

Figure 3: Analysis of pelleted VLPs.
OST-7 cells grown in parallel in 3.5cm culture dishes were infected with vTF7-3 and transfected after 1h with one or more of the plasmids pTUMM, pTUMS, pTM5ab and pTFMS. From 2h p.i. they were incubated at 32°C. Cells were radiolabeled with ^{35}S-labeled amino acids after which the culture supernatants were cleared by low-speed centrifugation, layered on top of a 25% sucrose cushion in a SW50 tube and centrifugated overnight at 37,000 rpm and 4°C. Pellets were dissolved in Laemmli sample buffer and proteins analyzed by polyacrylamide gel electrophoresis. The combinations of the expressed proteins are indicated at the top. The positions of the M protein and of the MHV S and chimaeric S* proteins are indicated at the right.

Figure 4: Cocultivation fusion assay.
Parallel cultures of MVA-T7pol infected and uninfected BHK-21 cells were mock-transfected or transfected with different S constructs. At 5h p.i. fcwf-D cells were seeded onto the BHK-21 cells. The formation of syncytia was followed light microscopically. The pictures shown were taken at 24h p.i.. Results are shown of the following

expressions: wild-type MHV S (MHV-S); wild-type FIPV S (FIPV-S); chimaeric MHV/FIPV S* (S*); chimaeric MHV/FIPV S# (S#); mock-transfection (-DNA); mock-infection + mock-transfection (-MVA-DNA).

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CLAIMS

1. A virus-like particle derived from a coronavirus provided with a at least one biologically active protein or fragment thereof associated with the surface of said virus-like particle other than the natural ectodomain of any one protein of the original corona virus.

2. A virus-like particle derived from a coronavirus provided with a at least one biologically active protein or fragment thereof associated with the inside of said virus-like particle other than the natural endodomain of any one protein of the original corona virus.

3. A virus-like particle derived from a coronavirus provided with a at least one functional targeting means associated with the surface of said virus-like particle other than the natural spike protein of the original corona virus.

4. A virus-like particle derived from a coronavirus according to any of claims 1 to 3 wherein said particle is provided with a coronavirus genome wherein a gene or parts thereof have been deleted.

5. A virus-like particle derived from a coronavirus according to any of claims 1 to 3 wherein said particle is provided with a coronavirus genome wherein a foreign gene or parts thereof have been inserted.

6. A virus-like particle according to claim 4 or 5 which has been attenuated.

7. A virus-like particle according to any of claims 1 to 6 which is a gene delivery vehicle.

8. A virus-like particle according to any of claims 1 to 6 which is an antigen or epitope delivery vehicle.

9. A virus-like particle according to anyone of claims 1 to 8 in which said biologically active molecule or said targeting means is a protein or (poly)peptide

10. A virus-like particle according to claim 9 in which the targeting means is a membrane component.

11. A virus-like particle according to claim 10 in which the membrane component comprises a viral envelope protein.

12. A virus-like particle according to claim 11 in which the membrane component comprises at least a part of a spike protein or a derivative thereof.

13. A virus-like particle according to claim 12 in which the membrane component comprises at least a cell surface receptor molecule.

14. A virus-like particle according to claim 13 in which the receptor molecule comprises the CD4 molecule or a derivative thereof.

15. A virus-like particle according to claim 14 in which the targeting means comprises a molecule, or fragments thereof, selected from the group of microbial antigens, hormones, enzymes and toxins.

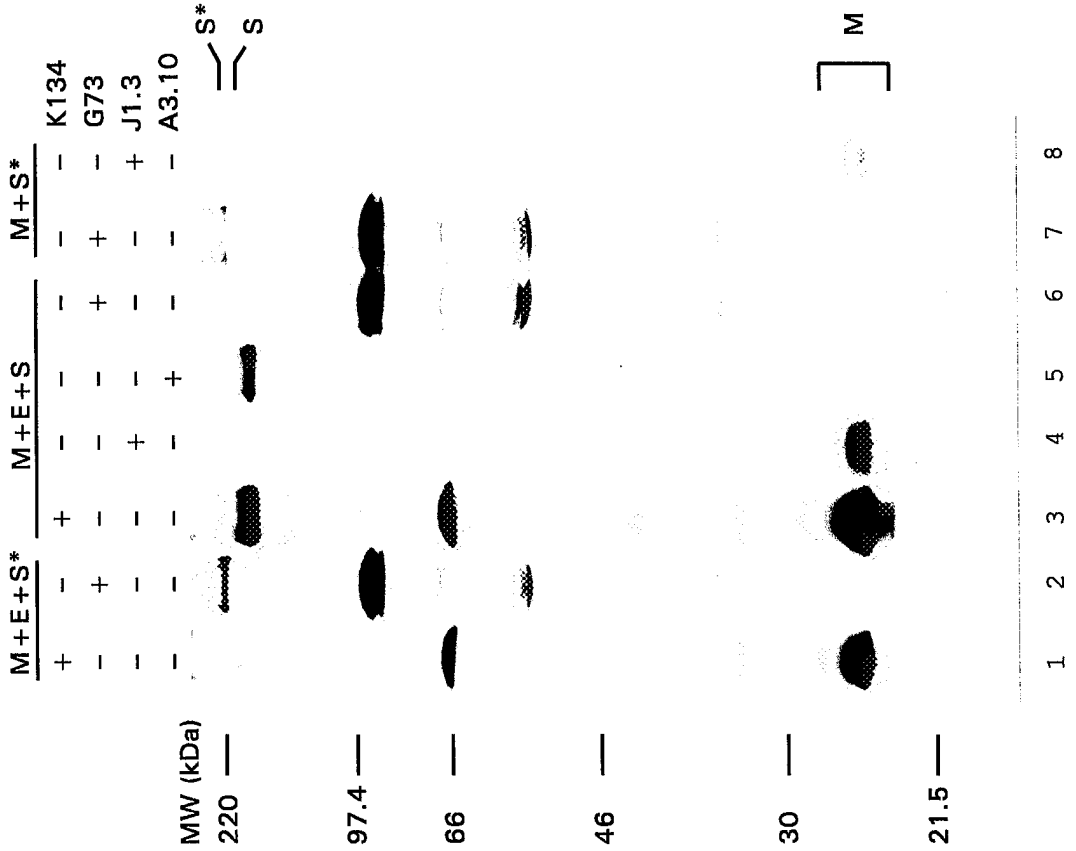
16. A virus-like particle according to claim 9 in which the targeting means comprises a molecule, or fragments thereof, selected from a single-chain antibody fragment.

17. A composition comprising a virus-like particle according to any of claims 1 to 16 for therapeutic use.

18. A composition comprising a virus-like particle according to any of claims 1 to 16 and a pharmaceutically acceptable carrier for use as an immunogen or vaccine.

19. A composition comprising a virus-like particle according to any of claims 1 to 16 for diagnostic use.

Fig. 1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66
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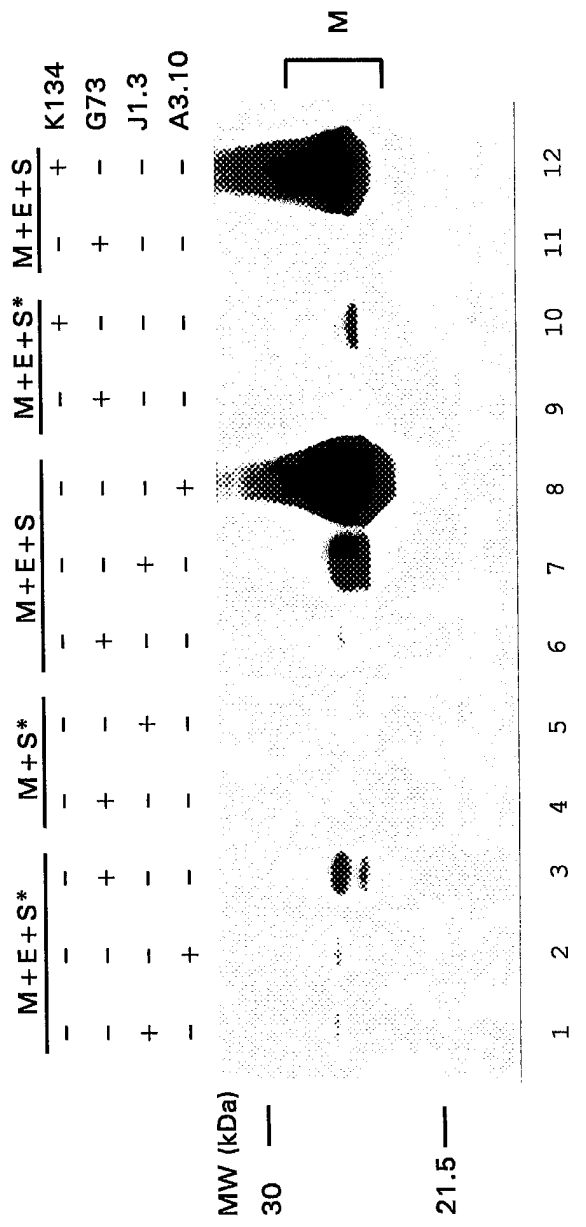
Fig. 2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M+E+S
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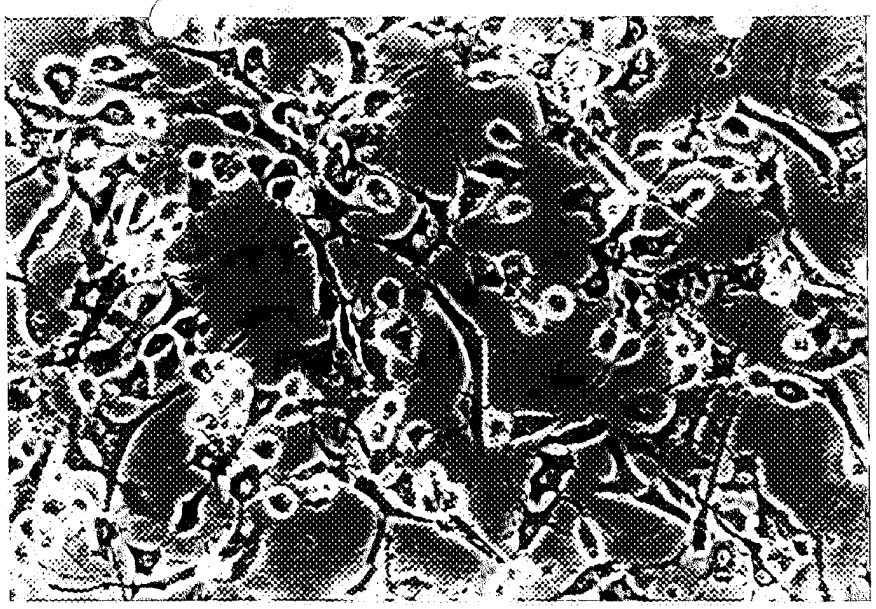
Fig. 3

S#



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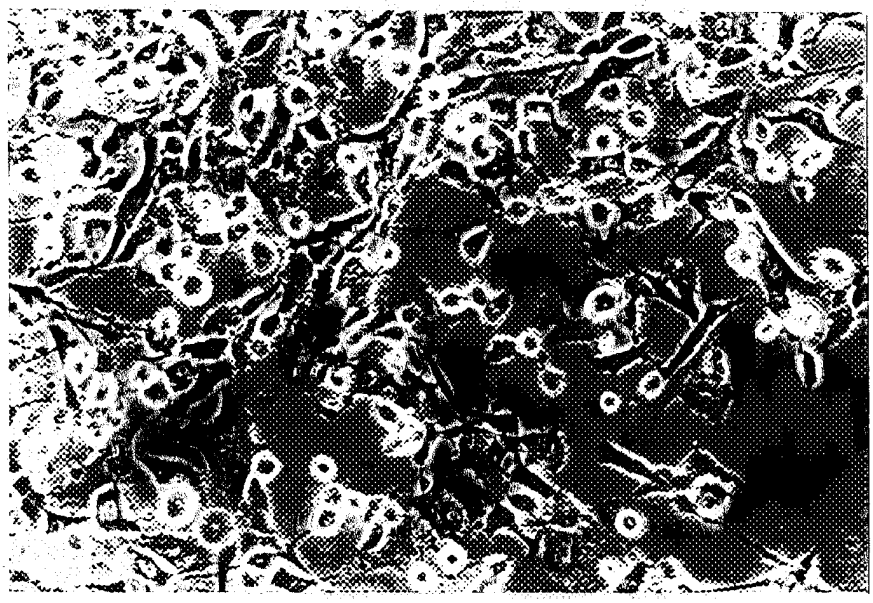

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-DNA

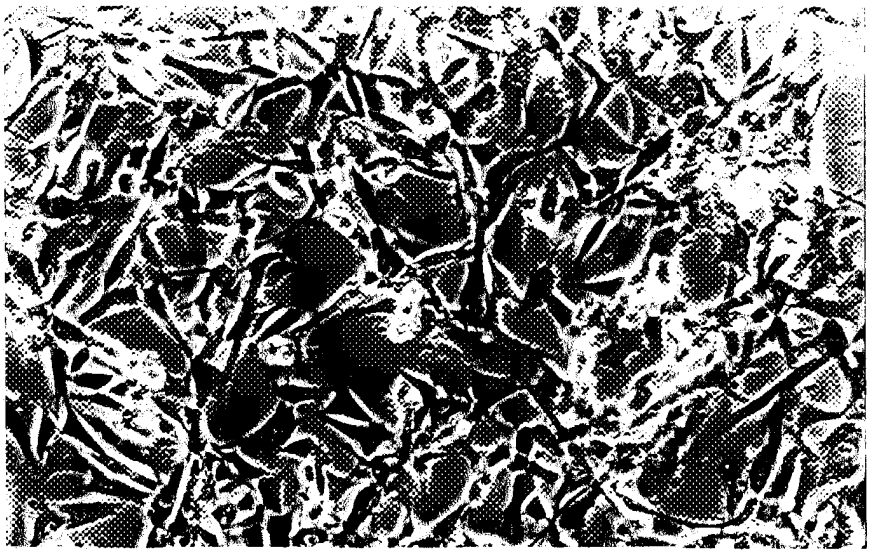
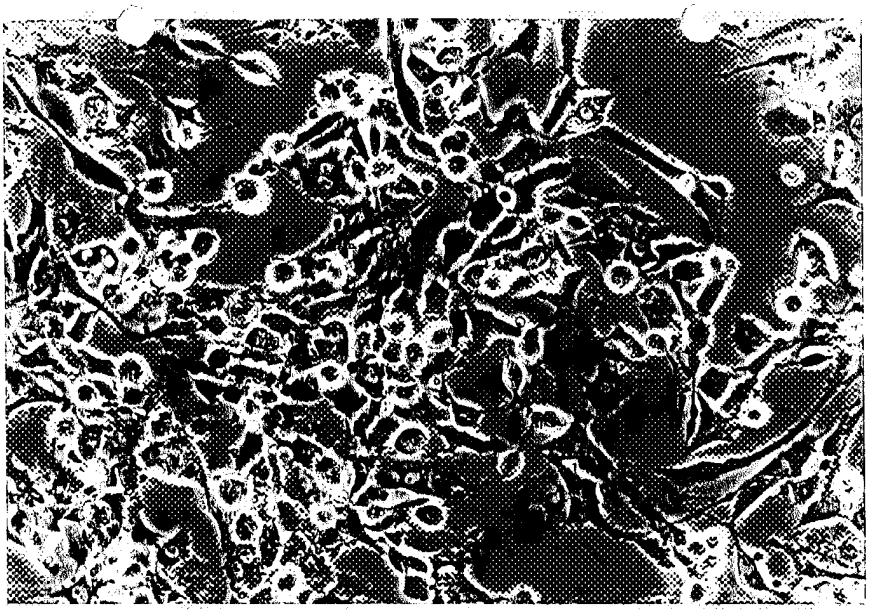
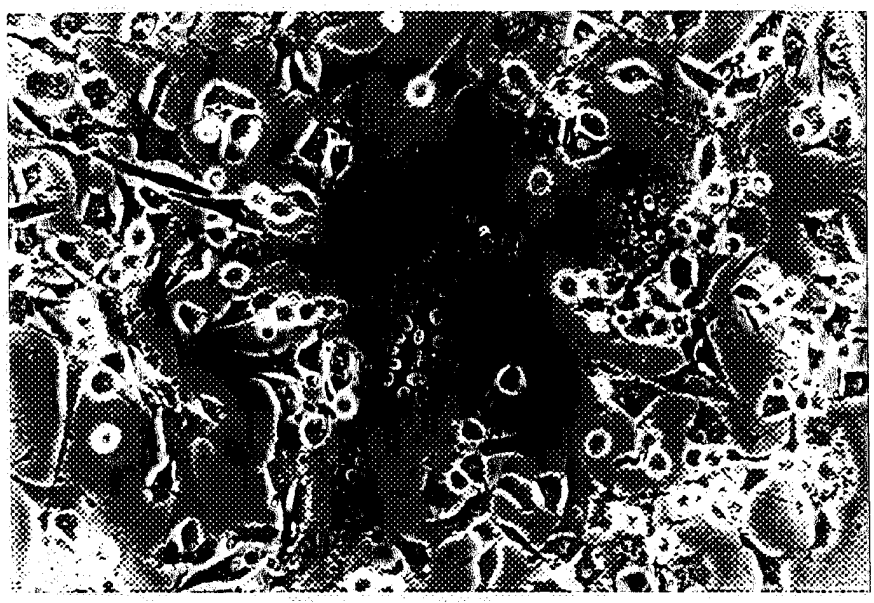


Fig. 4

MHV-S



FIPV-S



S*

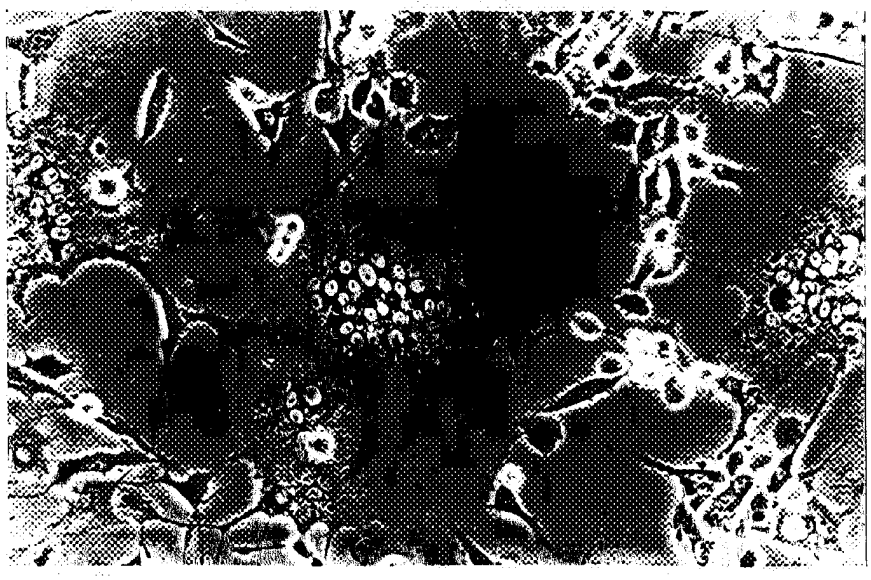


Fig. 4

INTERNATIONAL SEARCH REPORT

Interna. .al Application No
PCT/NL 98/00237

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/165 C12N7/04 C12N15/86

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	E.C.W. BOS ET AL.,: "The production of recombinant infectious DI-particles of a murine coronavirus in the absence of helper virus" VIROLOGY, vol. 218, 1996, pages 52-60, XP002043749 ORLANDO, FL, US see the whole document --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 September 1998	28/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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INTERNATIONAL SEARCH REPORT

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