Efficient production of F(ab')2 fragments in mammalian cells

Field of the invention

The invention relates to the field of recombinant protein production, more in particular to production of immunologically active antibody fragments in eucaryotic cells. The invention more in particular relates to the production of F(ab')2 fragments in eucaryotic cells.

Background of the invention

Results of recent clinical trials have generated much excitement and optimism for the potential benefits of fully human antibodies in the diagnosis and treatment of disease. This success is in part due to the technology of selecting antibodies against novel targets from phage display libraries, and also due to improved production platforms.

One IgG molecule comprises two heavy chains and two light chains. The heavy chains consist of (starting from the N-terminus): a variable region, a constant region, a hinge region and two additional constant regions (Figure 1). The hinge region contains cysteine residues that form disulphide bonds with a second heavy chain to mediate dimerisation of the protein. The number of cysteine residues varies depending on the IgG sub-class: IgG1 has 2 cysteines that form disulphide bonds in the hinge. The light chains consist of a variable region and a constant region; residues C-terminal to the constant region form disulphide bonds with residues immediately before the heavy chain hinge regions. Thus the four chains are held together by multiple disulphide bonds as well as other noncovalent interactions between the intimately paired chains.

The structure of an antibody may be defined as distinct domains: the Fc region which mediates effector functions and the F(ab') region which binds antigen (George and Urch, 2000). The two C-terminal constant domains of the heavy chains make up the Fc region. A F(ab') fragment comprises the light chain and the variable and first constant region of the heavy chain; a F(ab')2 fragment comprises two F(ab') fragments dimerised through the heavy chain hinge region (Figure 1).

Antibodies are under investigation as therapies for a wide range of clinical problems including organ transplantation, cardiac disease, infectious diseases, cancer, rheumatologic and autoimmune disease, neurologic disorders, respiratory diseases, as well as disorders with organs such as the blood, skin and digestive tract.

One of the major focuses for antibody discovery and development is in the field of cancer imaging and therapy (Carter, 2001). Antibodies may be used as naked molecules, or they may be labeled and so used as a magic bullet to deliver a cargo to the tumor (Borrebaeck and Carlsson, 2001; Park and Smolen, 2001). A number of naked antibodies are currently in the clinic. While it is clear that they are able to reduce tumor load in patients, the mechanism by which this occurs is unclear. Classically these might work by recruiting effector cells (via Fc receptors) or complement to the target cell. More recently it is becoming apparent that they may also function by binding cell surface proteins and then activating inappropriate signalling pathways or apoptotic signalling pathways, leading to cell death (Tutt et al., 1998).

Antibodies are currently used in the clinic both as intact IgG molecules and as F(ab') and F(ab')2 fragments. When choosing an antibody format there are several important issues. They

should have a high antigen avidity and specificity, be sufficiently small to penetrate tumor tissue and remain in the circulation long enough to localize to tumors. In addition (particularly if they are labeled with a radiolabel or other toxic moiety) they should clear from the body at a rate which prevents non-specific toxicity or high background.

F(ab')2 fragments exhibit a number of benefits over intact IgG related to the above points, which make them attractive for imaging and therapy. Firstly these molecules have a shorter half-life than intact IgG as they are more rapidly removed from the circulation by the kidneys as a result of their lower molecular weight, thus reducing potential toxicity (Behr et al., 1995). Another advantage of the reduced size is that they may penetrate tumor tissue and associated vasculature more readily (Yokota et al, 1992). In this way, more cells of the tumor mass are targeted.

There are also advantages due to the absence of the Fc region of the molecule. The F(ab')2 fragment does not induce activation of immune responses, as the Fc region (which binds complement and Fc receptors) is absent. This is of particular relevance in imaging studies, where a snap-shot of tumor dispersion and size only is required. F(ab')2 fragments also do not have the problem of non-specific binding to targets through the Fc moiety, and so background and non-specific labeling is reduced. The advantages listed above may also apply in part to F(ab') fragments. However F(ab')2 molecules are bivalent (as are intact IgGs) and so should bind target molecules with higher avidity. F(ab') fragments are monovalent, and as a result generally exhibit lower avidities. For these reasons, F(ab')2 fragments are highly desirable as clinical agents.

While these advantages of F(ab')2 fragments are clear, is has not proved as easy to make F(ab')2 fragments. There are several methods currently available. The classic method is to make the intact IgG, then digest it with a protease such as pepsin to remove the Fc region of the antibody. This has the problem that other regions of the molecule may be nicked by the protease (including the antigen binding region, resulting in the loss of binding capacity of the antigen-binding region), and digestion may not be complete. Further-purification is then required to remove the F(ab')2 fragment from non-digested antibody, the free Fc domain and the protease.

An alternative method is to make F(ab') fragments in bacteria and then dimerise the molecules to generate F(ab')2 molecules (Willuda et al., 2001; Zapata et al., 1995, Humphreys et al., 1998; US patent 5,648,237). Dimerisation may use specific self-associating peptides (which may prove antigenic *in vivo*), conjugation via chemical cross-linkers or *in vitro* reduction/oxidation of F(ab')-hinge fragments. These methods require additional purification steps and may produce unusual molecules (such as two F(ab') fragments linked "head-to-tail" so that the antigen binding regions are at opposite ends of the new molecule).

Another method for production of F(ab')2 fragments is to generate them directly in mammalian cells. While this might appear straightforward, it has been observed that F(ab') fragments are often produced in preference to F(ab')2 fragments. One report in which CHO cells were used for IgG4 F(ab')2 production indicated that F(ab')2 fragments accounted for only 10% of the protein produced, with F(ab') fragments accounting for 90% (King et al., 1992; King et al., 1994). The reason for this is unclear. Another important cell line in the

production of monoclonal antibodies is SP2/0; an attempt to produce IgG1 F(ab')2 fragments in this cell line yielded essentially only monovalent products. Addition of a IgG3 hinge, which comprises 11 sulphur bridges instead of the two sulphur bridges present in an IgG1 hinge, resulted in the production of 98% divalent product (Leung et al., 1999). However, increased numbers of sulphur bridges generally decrease production levels of the antibody fragments, and it is therefore preferable to have less sulphur bridges for production at a large scale. A similar picture was seen upon expression of F(ab')2 fragments in COS cells (De Sutter et al., 1992). Thus, despite these efforts, there is still a need for improved production methods of F(ab')2 fragments. PER.C6™ is a human cell line and an example of an immortalized primary eucaryotic host cell. It is able to grow in suspension culture in serum-free medium, which upon transfection with an appropriate expression vector and selection of stable cell lines, is capable of producing recombinant protein in abundance, as disclosed in WO 00/63403. In the '403 application it has been disclosed that PER.C6™ cells can express intact human IgG, but no specific data have been provided for F(ab')2 fragments.

In view of the above there is still a need for a recombinant expression platform which is capable of producing such fragments in sufficient yield without some of the disadvantages observed with the platforms of the prior art.

Summary of the invention

We demonstrate here that PER.C6™ cells, as an example of a eucaryotic immortalized primary cells, are capable of more efficiently producing and secreting F(ab')2 fragments without the need to take special measures disclosed in the prior art.

The F(ab')2 fragment so produced and secreted can bind antigen as efficiently as can intact IgG, while the monovalent F(ab') binds considerably less efficient.

More in particular, it was found, that the cell-line PER.C6™, and derivatives thereof, appear to be very suitable for the production of bivalent fragments of Ig-molecules and wherein the monovalent moieties making up the bivalent Ig-molecule fragment are linked via one or more disulphide bonds.

Thus, according to the invention, an immortalized primary eucaryotic host cell is provided comprising nucleic acid encoding an immunologically active bivalent multimeric antibody fragment, and/or a precursor thereof, functionally linked to sequences capable of driving expression of said fragments in said host cell when said cell is cultured under conditions allowing said expression. The invention provides a host cell comprising adenovirus E1 sequences, and further comprising recombinant nucleic acid encoding an immunologically active bivalent multimeric antibody fragment, and/or a precursor thereof, functionally linked to one or more sequences capable of driving expression of said fragment in said host cell. According to one preferred aspect of the invention, the immunologically active bivalent multimeric antibody fragment comprises an F(ab')2 fragment. The host cell preferably is a eucaryotic cell, more preferably a mammalian cell, even more preferably a human cell. In certain embodiments, a host cell according to the invention is derived from a retina cell, preferably a retina cell from a human embryo. In certain embodiments, the host cell is obtainable from a host cell chosen from the group consisting of 293 and PER.C6™ or progeny thereof. In the most preferred aspect of the invention, the host cell provided is obtainable from a PER.C6™ cell. According to a preferred aspect of the

invention, the host cell comprises a nucleic acid sequence encoding at least one E1 protein of an adenovirus or a homologue, fragment and/or derivative thereof, functional in immortalizing a primary cell when expressed in said cell. The invention also provides a PER.C6™ cell comprising nucleic acid encoding an F(ab')2 fragment. In a preferred embodiment of the invention, the sequence of driving expression comprises a region from a CMV promoter, more preferably the region of the CMV promoter comprises the CMV immediate early gene enhancer/promoter from nucleotide -735 to +95. In another aspect according to the invention said immunologically active bivalent multimeric antibody fragment is capable of selectively binding to activated vitronectin.

The invention also provides a host cell expressing and secreting immunologically active bivalent and monovalent antibody fragments, and/or precursors thereof, characterized in that the ratio of secreted bivalent active antibody fragment to monovalent active antibody fragment by said host cell is at least 1:3, wherein the two antigen binding regions of said bivalent active antibody fragment are not linked by peptide bonds.

The invention also provides a method of making a host cell capable of producing an immunologically active bivalent multimeric antibody fragment, the method comprising: introducing into an immortalized primary eucaryotic cell a nucleic acid sequence comprising a sequence encoding said antibody fragment or precursor thereof operably linked to a sequence capable of driving expression of said sequence encoding said antibody fragments in said cell.

The invention also provides a method of producing an immunologically active bivalent antibody fragment, comprising culturing a host cell according to the invention. In one

aspect, said method further comprises isolating and/or purifying said immunologically active bivalent antibody fragment.

The invention further provides a method of producing an immunologically active bivalent antibody fragment, wherein the two antigen binding regions of said immunologically active bivalent antibody fragment are not linked by a peptide bond, the method comprising: a) providing a host cell comprising adenovirus E1 sequences, said host cell further comprising a recombinant nucleic acid sequence comprising a sequence encoding said antibody fragment or precursor thereof operably linked to a sequence capable of driving expression of said sequence encoding said antibody fragment in said host cell; b) culturing said host cell; whereby said antibody fragment is secreted from said host cell, wherein the ratio of secretion of immunologically active bivalent to immunologically active monovalent antibody fragment is at least 1:3.

The invention also provides a method of producing an immunologically active bivalent antibody fragment, wherein the two antigen binding regions of said immunologically active bivalent antibody fragment are not linked by a peptide bond, the method comprising: a) introducing into an immortalized primary eucaryotic host cell a nucleic acid sequence comprising a sequence encoding said antibody fragment or precursor thereof operably linked to a sequence capable of driving expression of said sequence encoding said antibody fragment in said host cell; b) culturing said host cell; whereby said antibody fragment is secreted from said host cell, wherein the ratio of secretion of immunologically active bivalent to immunologically active monovalent antibody fragments is at least 1:3. According to another aspect of the invention, the method further comprises: c) isolating and/or

purifying said immunologically active antibody fragment. In the method according to the invention the two antigen binding regions of said immunologically active bivalent antibody fragment preferably are linked by one to ten sulphur bridges, more preferably by one or two sulphur bridges. In one preferred aspect the region of said immunologically active bivalent antibody fragment that is linked by said sulphur bridges is not derived from an IgG3. In the method according to the invention, said immunologically active bivalent antibody fragment preferably comprises an F(ab')2 fragment. According to yet another embodiment of the methods according to the invention, the host cell is a mammalian cell, more preferably a human cell. In other embodiments, said host cell is derived from a retina cell. In other embodiments, the host cell is chosen from 293 and PER.C6™. In the most preferred aspect of the invention, the host cell is a PER.C6™ cell. According to other preferred aspects of the invention, the ratio of secretion of immunologically active bivalent to immunologically active monovalent antibody fragments is at least 1:1, more preferably at least 2:1, and still more preferably at least 3:1. The host cell according to the method of the invention preferably comprises a sequence encoding at least one E1 protein of an adenovirus or a functional homologue, fragment and/or derivative thereof. In yet another embodiment of the method according to the invention, an immunologically active bivalent antibody fragment is capable of selectively binding to activated vitronectin.

The invention also provides a method for obtaining an F(ab')2 fragments, said method comprising: a) introducing into a eucaryotic cell a nucleic acid sequence encoding said fragment, or precursor thereof, operably linked to sequences capable of driving expression of said sequence encoding said

fragment in said cell; b) culturing said cell; whereby said fragment is secreted from said cell, wherein the ratio of secretion of F(ab')2 to F(ab') fragments is at least 1:3; c) isolating and/or purifying said F(ab')2 fragment; characterized in that said method is essentially devoid of a protease step.

In one embodiment of the invention said immunologically active antibody fragment comprises an amino acid sequence that is derived from or immunologically similar to an amino-acid sequence for a type of antibody fragment of the species said mammalian cell is obtained or derived from. An advantage is that said produced antibody fragment can be post-translationally modified according to the modification pattern of said species thereby allowing for a type of modification that is similar to the "natural" situation. Preferably, said species is a human. It has been observed that particularly for human and other human-like species such as monkeys the ratio of produced (human or human-like) dimeric versus monomeric antibody fragment is particularly favorable when said immunologically active antibody fragment comprises an amino acid sequence that is derived from or immunologically similar to an amino acid sequence for a type of antibody fragment of the corresponding species.

The invention also provides an F(ab')2 fragment obtainable by expression of said fragment in a cell derived from a PER.C6™ cell.

The invention further provides a crude preparation of an immunologically active antibody fragment obtainable by methods according to the invention. In a preferred embodiment of the invention, said immunologically active antibody fragment comprise an F(ab')2 and an F(ab') fragment. The invention also

provides an F(ab')2 fragment obtainable by separating an F(ab')2 fragment from F(ab') fragments in said crude preparation. In one preferred embodiment such F(ab')2 fragments can selectively bind to activated vitronectin. The invention also provides an F(ab')2 fragment that can selectively bind to activated vitronectin.

The invention also provides a pharmaceutical composition comprising an immunologically active antibody fragment according to the invention.

The invention also provides a pharmaceutical composition comprising an F(ab')2 fragment according to the invention, and a pharmaceutically acceptable carrier.

In another aspect the invention provides a composition comprising immunologically active bivalent and monovalent antibody fragments in a ratio of at least 1:3, wherein said fragments are produced by a host cell according to the invention. In one preferred embodiment, said ratio is at least 3:1.

The invention further provides a composition comprising immunologically active bivalent and monovalent antibody fragments in a ratio of at least 1:3, wherein said fragments are obtainable by a method according to the invention.

The invention also provides a vector useful in a method according to the invention, said vector comprising: a) DNA encoding VH1, CH1 and hinge region of an antibody, comprising introns, operably linked to a CMV promoter and a bovine growth hormone polyadenylation signal; b) DNA encoding VL and CL region of an antibody, comprising an intron, operably linked to a CMV promoter and a bovine growth hormone polyadenylation signal, wherein said CMV promoter comprises nucleotides -735 to +95 from the CMV immediate early gene enhancer/promoter.

In a preferred embodiment said antibody binds to activated vitronectin.

The invention also provides a plasmid designated pcDNA3002(Neo) as deposited under number 01121318 at the ECACC on December 13, 2001.

Detailed description of the invention

The invention provides a host cell comprising adenovirus E1 sequences, and further comprising recombinant nucleic acid encoding an immunologically active bivalent multimeric antibody fragment, and/or a precursor thereof, functionally linked to one or more sequences capable of driving expression of said fragment in said host cell. Methods of producing an immunologically active bivalent antibody fragment are also provided, comprising culturing a host cell according to the invention.

Host cell, immortalized primary cell

Host cells for recombinant protein production are known in the art. In one aspect, the host cell of the invention comprises adenovirus E1 sequences, preferably by comprising a nucleic acid sequence encoding at least one E1 protein of an adenovirus or a homologue, fragment and/or derivative thereof. These may be functional in immortalizing a primary cell when expressed in said cell. Said E1 protein may comprise the E1A protein, that function in transforming and immortalizing the host cell. Furthermore, the E1B protein of adenovirus may be expressed, which can repress apoptosis of the host cell. In one aspect therefore, the host cell of the invention comprises at least part of the E1 region of an adenovirus, comprising E1A and E1B sequences in expressible format.

Immortalized cells are known in the art, and can in principle grow indefinitely, in contrast to primary cells that will die after a limited number of cell divisions. Various tumor cell lines known in the art, including but not limited to cell lines such as chinese hamster ovary (CHO) cell lines, HeLa, baby hamster kidney (BHK), hybridoma cell lines including NS0

and Sp2-0, are also immortalized. A primary cell as meant herein is a cell that is not derived from a tumor. To be able to grow indefinitely, a primary cell needs to be immortalized in some kind. Immortalization of primary cells can for instance be achieved by introduction of the E1 region of an adenovirus in expressible form into the cells. Other possible methods include introduction of human papillomavirus (HPV) E6 and E7 sequences into the cells, introduction of SV40 T antigen into the cells, mutation of endogenous p53 of the cells, transfection with c-myc and a mutant p53 gene in the cells. A preferred cell line for use as a host cell according to the present invention, PER.C6™, was obtained as described e.g. in US patent 5,994,128; as described in that patent, PER.C6™ cells have been deposited at the ECACC under no. 96022940. Briefly, human embryonic retinoblasts were immortalized by introduction of the E1 region comprising E1A and E1B of adenovirus, wherein the E1A gene is driven by the human PGK promoter.

Immunologically active

Immunologically active in accordance with the invention means capable of selectively binding to an antigen, wherein selective binding is defined as binding with an affinity (Kd) of at least 5 * 10E4 liter/mole, more preferably 5 * 10E5, more preferably more than 5 * 10E6, still more preferably 5 * 10E7, or more. Typically, monoclonal antibodies may have affinities which go up to 10E10 liter per mole, or even higher.

Bivalent multimeric antibody fragment

IgGs typically have two identical Fab regions, *i.e.* two regions that bind antigen, and are therefore said to be bivalent. An antibody fragment according to the invention is

meant to define a molecule, which retains the binding function but lacks the region that mediate the effector functions. Accordingly, antibody fragments according to the invention lack at least the C-terminal constant domain of the heavy chain, more preferably antibody fragments according to the invention lack both the C-terminal constant domains of the heavy chain (C2 and C3). A bivalent antibody fragment according to the invention is a fragment, which comprise two binding regions. Each binding region comprises a light chain and a heavy chain fragment, which binding region may exist as a dimer (*i.e.* the heavy chain fragment and the light chain are bound to form a dimer) or as a monomer (*e.g.* in a single chain format (scFv)). The bivalent antibody fragments according to the invention are dimeric in the sense that the two binding regions making up the bivalent antibody fragment are linked to each other, although not via peptide bonds. The binding regions may dimerise in different ways, e.g. through one or more cystein dependent S-bridges (sulphur bridges), such as in the hinge regions directly attached to the C1 region of the two heavy chains. The at least two independent antigen binding sites may both bind the same or each bind different antigens. Monovalent antibody fragments as meant in the present invention have only one antigen binding site. One non-limiting example of a monovalent antibody fragment is a F(ab') fragment. The two binding regions may or may not be identical, and may or may not be monospecific, meaning that both binding regions recognize the same epitope with the same affinity, whereas the former means it may recognize the same epitope with different affinity or different epitopes.

A preferred bivalent dimeric antibody fragment according to the invention is a F(ab')2 fragment, which consists of two identical F(ab') fragments attached to each other through the

heavy chain hinge regions by one or usually two sulphur bridges (Figure 1).

A conjugated antibody fragment

The antibody fragments produced may be labeled or conjugated with any moiety (radio- or fluorescent label, toxin, protein or other agents) in the same way as intact antibodies may be labeled. Labeling may also take the form of generation of a fusion protein in the cell-line. Thus antibody fragments may also be conjugated to other polypeptides, which conjugates or immunoconjugates are meant to be included in this invention. It is also possible to generate bispecific fragments, with antigen binding sites situated either N-terminal or C-terminal to the hinge region.

Radiolabels may be used in both imaging and therapy. These may be alpha particle emitters such as Bismuth212 or Astatine211; these have a very high energy but only a small range, so bystander effects are minimal. Beta particle emitters are more commonly in use; these include Iodine131, Yttrium90 and Rhenium186 amongst others. These have a lower particle energy but a greater range, thus potentially causing bystander cell death in a tumor mass. In addition, antibodies may be labeled with cytotoxic moieties that kill the tumor cell upon internalization. An antibody linked to a bacterial toxin, calicheamicin, is currently on the market (Mylotarg; Carter, 2001). Other toxic moieties include maytansinoids (TAPs; ImmunoGen), as well as immunoliposomes loaded with chemotherapeutic agents amongst others. Cargo may also be any number of proteins, peptides, drugs or pro-drugs. It may also consist of a moiety to which a subsequently introduced therapeutic may bind.

There is also the potential to generate bispecific antibodies: one antigen binding domain can bind a tumour cell, the other a cell surface protein of an effector cell, thus inducing killing of the target cell. Homodimerisation of fragments may also yield proteins with enhanced anti-tumour characteristics.

Antibody fragment precursor

Proteins can be encoded by precursor proteins that require peritranslational and/or posttranslational modifications before the mature protein form is reached. Nucleic acid encoding precursor forms of antibody fragments, as well as the encoded precursor proteins themselves, including but not limited to preproteins containing secretion signals and the like, are included in the invention. The nucleic acid sequences encoding the fragments of interest can comprise introns or not. Similarly, it may be a cDNA or cDNA-like nucleic acid, or a genomic fragment, or combinations thereof.

Sequences capable of driving expression

To obtain expression of nucleic acid sequences encoding antibody fragments or precursors thereof, it is well known to those skilled in the art that sequences capable of driving such expression have to be functionally (also called operably) linked to the nucleic acid sequences encoding the antibody fragments or precursors thereof. Functionally linked is meant to describe that the nucleic acid sequences encoding the antibody fragments or precursors thereof is linked to the sequences capable of driving expression such that these sequences can drive expression of the antibodies or precursors thereof. Functionally linked includes but is not limited to direct linkage. A non-limiting example of functional linkage is for instance found in expression cassettes. Sequences

driving expression may include promoters, enhancers and the like, and combinations thereof. These should obviously be capable of functioning in the host cell, thereby driving expression of the nucleic acid sequences that are functionally linked to them. Promoters can be constitutive or regulated, and can be obtained from various sources, including viruses, procaryotic, or eucaryotic sources, or artificially designed. These nucleic acid sequences are obtainable by standard techniques that are well known in the art. Expression of nucleic acids of interest may be from the natural promoter or derivative thereof or from an entirely heterologous promoter. Some well-known and much used promoters comprise promoters derived from viruses, such as adenovirus, such as the adenovirus E1A promoter, promoters derived from cytomegalovirus (CMV), such as the CMV immediate early (IE) promoter, or derived from eucaryotic cells, such as methallothionein (MT) promoters, elongation factor 1α (EF-1α) promoter. Any promoter or enhancer/promoter capable of driving expression of the sequence of interest in the host cell is thus suitable in the invention. In one preferred embodiment the sequence capable of driving expression comprises a region from a CMV promoter, more preferably the region comprising nucleotides -735 to +95 of the CMV immediate early gene enhancer/promoter (CMVlong in Fig.2). This region comprises a very strong enhancer (Boshart et al, 1985). We have found that this CMVlong works particularly well, resulting in several fold higher expression in comparison to the use of the shorter, regular CMV promoter as present in e.g. the pcDNA3.1 plasmids (Invitrogen).

Culturing a cell is done to enable it to metabolize, and/or grow and/or divide. This can be accomplished by methods well known to persons skilled in the art, and includes but is not limited to providing nutrients for the cell. The methods comprise growth adhering to surfaces, growth in suspension, or combinations thereof. Several culturing conditions can be optimized by methods well known in the art to optimize protein production yields. Culturing can be done for instance in dishes, roller bottles or in bioreactors, using batch, fed-batch, continuous systems, hollow fiber or other methods, all meant to be included in the invention. In order to achieve large scale (continuous) production of recombinant proteins through cell culture it is preferred in the art to have cells capable of growing in suspension, and it is preferred to have cells capable of being cultured in the absence of animal- or human-derived serum or animal- or human-derived serum components. Thus isolation is easier and safety is enhanced due to the absence of additional animal or human proteins derived from the culture medium, while the system is also very reliable as synthetic media are the best in reproducibility.

In a preferred embodiment of the invention, an immunologically active bivalent multimeric antibody fragment is a F(ab')2 fragment. Expression of such fragments in eucaryotic cells has thus far been found to be problematic, as mostly F(ab') fragments were produced in the described systems. We provide here an immortalized primary eucaryotic host cell that is capable of expressing such F(ab')2 fragments in a functional manner in significant amounts.

Host cells obtainable from mentioned cells are derived from the mentioned cells, for instance by introducing nucleic acid

sequences into the mentioned cells. This can be achieved by any method known in the art to introduce nucleic acid into cells, such as for instance transfection, lipofection, electroporation, virus infection, and the like. The method used for introducing nucleic acid sequences in cells is not critical for the current invention. Said nucleic acid can be present in the cells extrachromosomally or stably integrated in the genome of said cells. Said cells are capable of driving expression transiently, but preferably said cells can drive expression in a stable manner. Alternatively, expression can be regulated.

According to a preferred aspect of the invention, the host cell provided is a mammalian cell, more preferably a human cell, more preferably a human cell that is obtainable from the group consisting of 293 cells and PER.C6™ cells. PER.C6™ is a human cell line capable of expressing proteins in a highly reproducible, upscalable manner, as disclosed in WO 00/63403. This invention discloses that PER.C6™ cells are capable of producing immunologically active bivalent multimeric antibody fragments when nucleic acid encoding such fragments functionally linked to sequences capable of driving expression of said fragments is present in the cells. Thus, according to one preferred aspect of the invention, the host cell is obtainable from a PER.C6™ cell.

The host cells of the present invention can be immortalized primary cells. Nucleic acid encoding E1 protein of an adenovirus can immortalize cells, and host cells according to the invention may be immortalized by the presence of said E1 nucleic acid sequence, such as is the case for instance in PER.C6™ cells. Other embryonic retinal cells as well as

amniocytes that have been immortalized by E1, can be useful in the present invention. Transformed human 293 cells (of embryonic kidney origin, cells also known as HEK293 cells) also have been immortalized by the E1 region from adenovirus (Graham et al., 1977), but PER.C6™ cells behave better in handling than said 293 cells. PER.C6™ cells have been characterized and documented extensively, while they behave significantly better in upscaling, suspension growth and growth factor independence. Especially the fact that PER.C6™ cells can be brought in suspension in a highly reproducible manner makes it very suitable for large scale production. Another advantage of the presence of adenovirus E1 region nucleic acid as compared to cells lacking this sequence in the invention is that adenovirus E1A as a transcriptional activator is known to enhance transcription from certain promoters, including the CMV IE enhancer/promoter (Gorman et al., 1989; Olive et al., 1990). Thus, when the recombinant protein to be expressed is under the control of the CMV enhancer/promoter, as in one of the preferred embodiments of the invention, expression levels of the recombinant protein increase in cells comprising E1A. Therefore, one aspect of the invention provides host cells comprising a nucleic acid sequence encoding at least one E1 protein of an adenovirus or a homologue, fragment and/or derivative thereof functional in immortalizing a primary eucaryotic cell.

The immunologically active bivalent multimeric antibody fragments can bind to any chosen antigen target. Methods to identify such targets and discover antibodies or antibody fragments to such targets are well known in the art. Preferably, antibodies or antibody fragments are of human origin, but obviously antibody fragments or antibodies can be

of other origins as well. As an example, the present invention discloses production of F(ab')2 fragments that selectively bind to activated vitronectin.

An immunologically active bivalent antibody fragment, exemplified by a F(ab')2 fragment, can be produced in several ways, but as discussed, there is still a need for improved production methods of F(ab')2 fragments. It would be particularly useful if eucaryotic host cells and methods for production of such fragment would be found that are characterized in improved ratios of produced F(ab')2 fragments over F(ab') fragments, or generally improved ratios of immunologically active bivalent over monovalent antibody fragments. Thus, the present invention provides for the first time a host cell expressing and secreting an immunologically active bivalent and monovalent antibody fragment, and/or precursor thereof, characterized in that the ratio of secreted bivalent immunologically active antibody fragment to monovalent immunologically active antibody fragment by said host cell is at least 3:1, wherein the two antigen binding regions of said bivalent active antibody fragment are not linked by peptide bonds. It will be understood by the skilled person that higher ratios are preferable. An example where this ratio is even higher than 3:1 is disclosed in the present invention.

According to the invention it was shown that it is possible to recombinantly produce bivalent multimeric antibody fragments and monovalent antibody fragments in a ratio of at least 1:3. Use of a hinge region that is derived from IgG3 in F(ab')2 fragments has been described to result in better ratios of F(ab')2 to F(ab') fragments when compared to a hinge region

derived from IgG1 (Leung et al., 1999). This is likely due to the high number of sulphur bridges (11 potential sulphur bridges) linking the two antibody binding regions in an IgG3 hinge. However, increased numbers of sulphur bridges generally decrease production levels of the antibody fragments, and it is therefore preferable to have less sulphur bridges for production at a large scale. This invention provides for a host cell and methods capable of expressing immunologically active bivalent and monovalent antibody fragments, of which the two antigen binding regions are linked by only few sulphur bridges, in a much better ratio than achieved with the host cells and methods described till now.
Therefore, in another embodiment of the invention, the two antigen binding regions of said immunologically active bivalent and monovalent antibody fragments are linked by one to ten sulphur bridges, more preferably by one or two sulphur bridges. According to one preferred aspect, said immunologically active bivalent fragments are F(ab')2 fragments.

Culturing the cells of the invention can be done according to a variety of ways, generally known and described in the art. Optimization of culturing conditions can be done to improve the yields of produced antibody fragments, and to improve the ratio of secreted immunologically active bivalent multimeric to monovalent antibody fragments. Such optimization may include but is not limited to the growth media and additives, culturing temperature, time of growth and production phases, culture dish or bioreactor type and volume, and the like. Preferably, growth media lacking animal- or human-derived serum or animal- or human derived serum components are used, such as e.g. ExCell 525 (JRH) medium. Defined culture media

are highly controllable, and impose less safety issues and high reproducibility. They also are highly beneficial for downstream processing steps, when the desired proteins or protein fragments are to be isolated.

In a preferred embodiment of the invention, the said immunologically active antibody fragments are isolated and/or purified. Any step that improves the ratio of the desired product to any byproducts can be used to achieve this, and is meant to be included in the invention. Many methods are known in the art for isolating and/or purifying, and some non-limiting examples are filtration, centrifugation, chromatography, including for instance affinity-chromatography, hydrophobic interaction, size-fractionation, anion-exchange, cation-exchange, and the like. In the example, size-fractionation is performed to separate the F(ab')2 from the F(ab') fragments.

Immunologically active bivalent antibody fragments, exemplified by F(ab')2 fragments, can be produced by protease (e.g. pepsin) digestion of complete antibodies, but as discussed this method has several disadvantages. Here we provide a method that is capable of producing large quantities of functional bivalent antibody fragments without the use of protease steps.

It is another aspect of the present invention to provide F(ab')2 fragments obtainable by expression of said fragments in a cell derived from a PER.C6™ cell. Such F(ab')2 fragments are efficiently obtainable in high amounts.

According to another aspect of the invention a crude

preparation of an immunologically active antibody fragment is provided, obtainable by methods according to the invention. A crude preparation may include the culture medium used to culture the cells, and any preparation comprising the immunologically active antibody fragments somewhere in the process of purifying the desired material. In a preferred embodiment, said immunologically active antibody fragments comprise F(ab')2 and F(ab') fragments. In another preferred aspect of the invention, the F(ab')2 fragments obtainable by separating the F(ab')2 fragments from F(ab') fragments in the crude preparation are provided. Separation can be done according to any method known in the art, including but not limited to size exclusion chromatography, affinity chromatography, anion- and/or cation-exchange, centrifugation, filtration. It will be clear to the skilled person that separation methods based on mass or size of the fragments are particularly useful for this purpose.

Also included as an aspect of the invention are pharmaceutical compositions comprising an immunologically active antibody fragment, preferably a F(ab')2 fragment, according to the invention. Pharmaceutical compositions may include or not carriers for administration of the desired material to humans or animals. Such carriers can include but are not limited to salts, sugars, proteins, lipids. The administration of pharmaceutical compositions can be done in a variety of ways, which are not critical for the present invention. One non-limiting example for the use of pharmaceutical compositions according to the present invention comprises the administration of a F(ab')2 fragment with a desired specificity in the form of a pharmaceutical composition to a human for imaging purposes, for instance to locate a tumor

that is selectively bound by said F(ab')2 fragment. Administration of F(ab')2 fragment producible according to the invention for therapeutic purposes is also possible.

It is another aspect of the invention to provide a vector useful in a method according to the invention. The vector will comprise DNA encoding VH1, CH1 and hinge region of an antibody, optionally comprising introns. VH1 is the variable region of the heavy chain, and CH1 is the first constant region of the heavy chain as present in complete IgG. The hinge region is situated just behind the CH1 region and is generally the part where the two antigen binding sites of an antibody are linked via cystein-dependent sulphur bridges. The vector also will comprise DNA encoding VL and CL region of an antibody, optionally comprising an intron. VL and CL are the variable and constant region of the light chain, respectively. A vector is a DNA sequence capable of replicating in host cells, and can but need not be a plasmid, phagemid, phage, yeast artficial chromosome, and the like. It usually comprises at least a sequence responsible for replicating the DNA in a host. Preferably said vector also comprises selectable marker DNA, conferring to the host cell in which the vector is present the ability to grow in the presence of a toxic substrate or in the absence of an otherwise essential growth factor. Preferably said vector comprises replication signals for propagation in a microbial host, so it can easily be obtained in quantities that are practical. The region comprising DNA encoding VH, CH1 and hinge region (heavy chain fragment) may be of genomic origin, or can be cDNA in which artificial introns are optionally present. The same holds for the region comprising DNA encoding VL and CL (light chain fragment). DNA of said VH, CH and hinge as well as for VL and

CL should be in such a constellation that both a heavy and a light chain transcription product is formed which upon splicing can be translated into functional protein comprising a heavy and a light chain fragment, respectively. DNA encoding precursors of said fragments is meant to be included in the invention. The heavy and light chain fragment encoding DNA regions preferably encode human antibody fragments. Other protein encoding DNA such that an immunofusion protein is encoded may or may not be present. DNA encoding the heavy chain fragment and DNA encoding the light chain fragment can each be operably linked to a CMV promoter, such that said CMV promoter is capable of driving transcription of said DNA in a suitable cell, described in the invention. The CMV promoter is a strong promoter useful for driving expression of recombinant proteins to high levels, as for instance described in US patent no. 5,186,062. The CMV promoter as used herein comprises nucleotides -735 to +95 of the major immediate early gene enhancer/promoter, wherein the nucleotides are numbered relative to the transcriptional start site. This region comprises the enhancer and promoter of the CMV immediate early promoter, and the person skilled in the art may make substitutions, insertions and/or deletions in this region, and test the promoter for usefulness according to methods known in the art, and use such a mutated CMV enhancer/promoter according to the invention without departing from the scope of the invention. The use of this promoter further has the advantage that E1 sequences as present in host cells according to the invention can augment transcription rates from this promoter. Furthermore, both DNA encoding the heavy chain fragment and the light chain fragment can be operably linked to a polyadenylation signal, preferably from the bovine growth hormone gene. Such a signal is useful for conferring good

polyadenylation of the transcripts, resulting in better translation and hence in higher recombinant product yields (as e.g. disclosed in US patent no. 5,122,458). In the present invention, we have reduced to practice an example where the antibody fragments encoded by the vector can form F(ab')2 fragments that selectively bind to activated vitronectin. As obvious to the skilled person, the VH and VL regions of the vector can encode any VH and VL region, which determine the antigen specificity of the resulting antibody fragments. Methods to obtain DNA sequences encoding VH and VL comprising regions of interest are known to those in the art. These include but are not limited to obtaining such DNA from hybridoma cells or for instance by phage-display. Said fragments can then be cloned by standard techniques either directly or via extra steps into the vector of the invention. The vector provided in the invention is particularly useful for use in the host cells of the invention, as we found that it can give rise to high expression levels of the desired proteins.

The invention also provides a plasmid useful for expression of recombinant proteins in host cells in general, plasmid pcDNA3002(Neo) fig. 2, deposited on December 13, 2001 at the European Collection of Cell Cultures (ECACC) under number 01121318. We have found this plasmid particularly useful for expression of multiple proteins and dimeric proteins, including immunoglobulins and fragments thereof in the host cells of the invention. The DNA encoding the protein(s) or fragments of interest can be cloned behind the CMVlong promoters in this plasmid, by standard techniques well known to persons skilled in the art.

To illustrate the invention, the following examples are provided, not intended to limit the scope of the invention. DNA encoding a complete antibody as well as the antibody fragments constituting a F(ab')2 fragment directed against activated vitronectin was cloned and expressed in PER.C6™ cells, expression was shown and after purification the vitronectin-binding activity of the resulting proteins was demonstrated.

Example 1. Construction of expression vectors.

An expression plasmid was generated which encodes both the light and heavy chains of an IgG1 antibody that recognizes activated vitronectin (as disclosed in EP 1130099). The DNA encoding the antigen binding region of this antibody was first isolated from a scFv phage display library and a leader sequence and constant regions were added prior to cloning into the expression vector pcDNA3002(Neo) (Figure 2). The expression vector pcDNA3002(Neo)is deposited on December 13, 2001 at the European Collection of Cell Cultures (ECACC) under number 01121318. The resulting plasmid is pVN18LcvHcv (Figure 3), which is deposited on December 13, 2001 at the ECACC under number 01121320. In addition a plasmid was constructed which contains an intact light chain and a heavy chain which is truncated immediately after the last translated codon of the hinge region. This was followed by a sequence encoding a 6 Histidine tag and then a stop codon. This plasmid, pVN18LcvHcvHis, can express a F(ab')2 fragment (Figure 3), and is deposited on December 13, 2001 at the ECACC under number 01121319. An additional plasmid has also been generated for F(ab')2 production that does not contain sequence encoding a

His-tag. Procedures were performed essentially as described in Sambrook and Russell, 2001.

Generation of pVN18LcvHcv and pVN18LcvHcvHis

Variable regions of the light and heavy chains of the anti-vitronectin antibody were isolated from a phage display library. These were separately cloned into vectors while also adding leader (signal) sequences at the N-termini, and constant regions at the C-termini of the two chains, resulting in plasmids VL VN18 and VH VN18 comprising the light and heavy chain respectively. The light chain of the anti-vitronectin IgG is a kappa type of chain. This will be present in both expression plasmids and so was inserted first into pcDNA3002(Neo). Plasmid VL VN18 was used as a template for PCR of the kappa light chain and to include convenient restriction sites (for general molecular cloning procedures, see e.g. Sambrook and Russel, 2001).

Oligo E001: CCT*GGCGCGCC*ACC**ATG**GCATGCCCTGGCTTCCTGTGG

This is homologous to the start of the translated sequence. The start codon is in bold; Kozak sequence is underlined; the AscI site used for cloning is in italics.

Oligo E002: CCGG*GTTAAC***TA**ACACTCTCCCCTGTTGAAGC

This is homologous to the non-coding strand at the end of the translated sequence. The stop codon is in bold; the HpaI site used for cloning is in italics.

Using these oligonucleotides the light chain was amplified using Pwo polymerase as a 1.3kb fragment, digested with AscI and HpaI and ligated into pcDNA3002(Neo) digested with the same enzymes. The resulting plasmid is pVN18Lcv (not shown).

Two forms of the heavy chain were then inserted into this plasmid to generate either the intact IgG1 molecule, or the

F(ab')2 fragment. Plasmid VH VN18 was used as a template for PCR amplification.

For production of the intact IgG1, the following oligonucleotides were used:

E003: GGA*GGATCC*GCCACC**ATG**GCATGCCCTGGCTTCCTGTGG

This is homologous to the start of the translated sequence. The start codon is in bold; Kozak sequence is underlined; the BamHI site used for cloning is in italics.

E004: GGATG*GCTAGC***TCA**TTTACCCGGAGACAGGGAGAG

This is homologous to the non-coding strand at the end of the translated sequence. The stop codon is in bold; the NheI site used for cloning is in italics.

Using these oligonucleotides the heavy chain was amplified using Pwo polymerase as a 2.2kb fragment, digested with BamHI and NheI and ligated into pVN18Lcv digested with the same enzymes. The resulting plasmid is pVN18LcvHcv.

For generation of the His-tagged F(ab')2 fragment, the following oligonucleotides were used to PCR the heavy chain fragment from VH VN18:

E003: GGA*GGATCC*GCCACC**ATG**GCATGCCCTGGCTTCCTGTGG

This is homologous to the start of the translated sequence. The start codon is in bold; Kozak sequence is underlined; the BamHI site used for cloning is in italics.

E005:

GGATG*GCTAGC***TCA**ATGGTGATGGTGATGATGTGGGCACGGTGGGCATGTGTGAGTT

This is homologous to the non-coding strand at the end of the translated sequence. The stop codon is in bold; the NheI site used for cloning is in italics and the sequence coding for six histidine residues forming the His-tag is underlined. Thus the amino acid sequence at the terminus of the heavy chain is:

GluProLysSerCysAspLysThrHisThrCysProProCysProHisHisHisHisHisHi
sStop

Using these oligonucleotides the heavy chain fragment was amplified from plasmid VH VN18 as a 1.3kb fragment using Pwo polymerase, digested with BamHI and NheI and ligated into pVN18Lcv digested with the same enzymes. The resulting plasmid is pVN18LcvHcvHis.

The sequences of the antibody-encoding regions are shown in Figures 8 - 10.

Example 2. Transfection of PER.C6™ cell lines and production of F(ab')2 fragments

Cells were transfected with either pVN18LcvHcv or pVN18LcvHcvHis by a lipofectamine based method. In brief, PER.C6™ cells were seeded at 3.5 x 10^6 cells per 96mm tissue culture dish. For each dish, 2µg plasmid DNA was mixed with 10µl lipofectamine (Gibco); this was added to the cells in serum free DMEM medium (total volume 7ml) and incubated for 5 hours. This was then replaced with complete medium. The following day (and for the ensuing 3 weeks) cells were grown in DMEM in the presence of 0.5mg/ml Geneticin (G418) to select for clones that were stably transfected with the plasmid. Clones secreting high levels of monoclonal into the cell culture supernatant were selected by an ELISA assay. In brief, wells of a 96-well plate were coated with antibody raised against Ig kappa light chain. After blocking with a milk solution, samples were added to wells at varying dilutions and incubated for 1 hour. After washing, detection antibody (biotin-labeled anti-IgG) was applied for 30 minutes. After a further washing step, this was detected by addition of streptavidin-horse radish peroxidase, followed by a final wash

and addition of substrate O-phenylene diamine dihydrochloride. Antibody concentration was determined by comparing optical density at 492nm with that of a known antibody standard. The top producing clones from each transfection (IgG or F(ab')2) were selected for further analysis. These were:

Intact IgG: clones 125 and 243

F(ab')2: clones 118 and 195

Production of antibody is performed in serum-free medium. Thus the adherent cells in tissue culture flasks were washed with PBS, then ExCell 525 medium (JRH) was added and the cells incubated for a further 4 days to allow secreted antibody to accumulate in the cell culture medium. A sample of the medium was electrophoresed on reducing SDS-PAGE (Figure 4). The heavy and light chains that comprise the intact, secreted antibody are the predominant protein species. The truncated heavy chain of the F(ab')2 fragment is present at a lower concentration than either the light chain or the intact heavy chain of IgG. The material was then purified: intact IgG over a Protein A column and F(ab')2 fragments over a Protein L column (Protein L binds kappa light chains). Purified material was dialyzed into PBS and then analyzed by gel filtration on a HiLoad 16/60 Superdex 200 column. The results of the gel filtration for clone 243 (intact IgG) and 118 (F(ab')2) are shown in Figure 5. The results are identical as those seen for clone 125 (IgG) and clone 195 (F(ab')2) for which data is not shown. Intact IgG runs as a single peak with elution time 67.18 (as expected for a protein of 150kDa). The F(ab')2 fragment runs as two main peaks; elution times suggest that the first is F(ab')2 and the second is F(ab'). This was confirmed by electrophoresis over non-reducing SDS-PAGE (Figure 5). Subsequent purifications indicated that the ratio of F(ab')2:F(ab') often is better. This is shown in Figure 6,

again for clone 118, where the percentage of F(ab')2 and F(ab') are 76% and 22% respectively. Similar results were also obtained with the constructs lacking a His-tag.

The efficiency of binding to antigen was determined for intact IgG, F(ab')2 and F(ab'). The antibody described above was raised against activated vitronectin, thus vitronectin was purified in this form (Yatohgo et al., 1988) and bound to the wells of a 96-well plate. Upon an ELISA analysis, the IgG and F(ab')2 material from two different clones bound equally efficiently whereas the F(ab') material bound the vitronectin approximately 100-fold less efficiently (Figure 7). This indicates that the F(ab')2 produced by this method does indeed bind antigen with equal avidity as the intact divalent IgG.

Brief description of the Figures.

Fig. 1. Intact IgG (left) and F(ab')2 fragment of IgG (right). Variable regions are pale, constant regions are dark. Disulphide bonds are marked in black.

Fig. 2. Expression vectors.

Fig. 3. Vectors for expression of intact IgG or F(ab')2 fragment of IgG.

Fig. 4. Reducing SDS-PAGE of cell culture supernatant from clones producing intact IgG and F(ab')2 fragment.
A: clone 243 IgG
B: clone 125 IgG
C: clone 195 F(ab')2
D: clone 118 F(ab')2

Fig. 5. Gel filtration analysis and non-reducing SDS-PAGE of purified samples of IgG and F(ab')2 material. Elution times are indicated above peaks.

Fig. 6. Additional example of gel filtration of F(ab')2 material. Elution volumes are indicated above peaks; F(ab')2 (72.89 mins) accounts for 76% of material; F(ab') (85.01 mins) accounts for 22% of material.

Fig. 7. Antigen binding of intact IgG (clones 243 and 125), F(ab')2 and F(ab') (clones 118 and 195). F(ab') binds vitronectin approximately 100X less efficiently than does IgG and F(ab')2.

Fig. 8. Complete DNA sequence encoding the light chain of the anti-vitronectin IgG1 (this sequence is identical in both pVN18LcvHcv and pVN18LcvHcvHis).

Fig. 9. Complete DNA sequence encoding the complete heavy chain of the anti-vitronectin IgG1.

Fig. 10. Complete DNA sequence encoding the trucated (F(ab')2) heavy chain fragment of anti-vitronectin IgG1, with His-tag sequence underlined.

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claims

1. A host cell for recombinant expression of a F(ab')2 fragment, said host cell comprising adenovirus E1 sequences, said host cell being derived from a human embryonic retina cell, said host cell further comprising recombinant nucleic acid encoding a F(ab')2 fragment, functionally linked to one or more promoter and/or enhancer sequences.

2. A host cell according to claim 1, wherein said host cell is derived from a PER.C6™ cell.

3. A host cell according to anyone of the preceding claims, wherein the one or more promoter and/or enhancer sequences comprise a region from a CMV promoter.

4. A host cell according to claim 3, wherein the region of a CMV promoter comprises the CMV immediate early gene enhancer/promoter from nucleotide -735 to +95.

5. A method of producing a F(ab')2 fragment, the method comprising:
a) providing a host cell comprising adenovirus E1 sequences and being derived from a human embryonic retina cell, said host cell further comprising recombinant nucleic acid encoding a F(ab')2 fragment, and/or a precursor thereof, functionally linked to one or more promoter and/or enhancer sequences;
b) culturing said host cell;
whereby said F(ab')2 fragment is secreted from said

host cell.

6. A method according to claim 5, further comprising:
c) isolating and/or purifying said F(ab')2 fragment.

7. A method according to claim 5 or 6, wherein the two antigen binding regions of said F(ab')2 fragment are linked by at least one and not more than ten sulphur bridges.

8. A method according to claim 7, wherein the two antigen binding regions of said F(ab')2 fragment are linked by one or two sulphur bridges.

9. A method according to any one of claims 5-8, wherein the region of said F(ab')2 fragment that is linked by said sulphur bridges is not derived from an IgG3.

10. A method according to any one of claims 5-9, wherein said host cell is a PER.C6™ cell.

11. A method according to any one of claims 5-10, further comprising formulating the F(ab')2 fragment into a pharmaceutical composition.