TITLE OF THE INVENTION

Human binding molecules capable of neutralizing influenza A viruses of phylogenetic group 1 and phylogenetic group 2 and influenza B viruses

FIELD OF THE INVENTION

The invention relates to medicine. The invention in particular relates to human binding molecules capable of neutralizing influenza A viruses of both phylogenetic group 1 and phylogenetic group 2. In particular, the invention relates to binding molecules capable of neutralizing influenza A viruses of both phylogenetic group 1 and phylogenetic group 2, as well as influenza B viruses. The invention further relates to the diagnosis, prophylaxis and/or treatment of an infection caused by influenza A viruses of phylogenetic groups 1 and 2, and preferably also influenza B viruses.

BACKGROUND OF THE INVENTION

Influenza infection (also referred to as "influenza" or "the flu") is one of the most common diseases known to man causing between three and five million cases of severe illness and between 250,000 and 500,000 deaths every year around the world. Influenza rapidly spreads in seasonal epidemics affecting 5-15% of the population and the burden on health care costs and lost productivity are extensive (World Healthcare Organization (WHO)).

There are 3 types of influenza virus (types A, B and C) responsible for infectious pathologies in humans and animals. The type A and type B viruses are the agents responsible for the influenza seasonal epidemics and pandemics observed in humans.

Influenza A viruses can be classified into influenza virus subtypes based on variations in antigenic regions of two genes that encode the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) which are required for viral attachment and cellular release. Currently, sixteen subtypes of HA (H1-H16) and nine NA (N1-N9) antigenic variants are known in influenza A virus. Influenza virus subtypes can further be classified by reference to their phylogenetic group. Phylogenetic analysis (Fouchier et al., 2005) has demonstrated a subdivision of HAs comprising two main groups (Air, 1981): *inter alia* the H1, H2, H5 and H9 subtypes in phylogenetic group 1 (herein also referred

to as "group 1") and *inter alia* the H3, H4 and H7 subtypes in phylogenetic group 2 (or "group 2"). Only some of the influenza A subtypes (i.e. H1N1, H1N2 and H3N2) circulate among people, but all combinations of the 16 HA and 9 NA subtypes have been identified in animals, in particular in avian species. Animals infected with influenza A often act as a reservoir for the influenza viruses and certain subtypes have been shown to cross the species barrier to humans, such as the highly pathogenic influenza A strain H5N1.

The influenza type B virus strains are strictly human. The antigenic variations in HA within the influenza type B virus strains are weaker than those observed within the type A strains. Two genetically and antigenically distinct lineages of influenza B virus are circulating in humans, as represented by the B/Yamagata/16/88 (also referred to as B/Yamagata) and B/Victoria/2/87 (B/Victoria) lineages (Ferguson et al., 2003). Although the spectrum of disease caused by influenza B viruses is generally milder than that caused by influenza A viruses, severe illness requiring hospitalization is still frequently observed with influenza B infection.

Current approaches to dealing with annual influenza epidemics include annual vaccination, preferably generating heterotypic cross-protection. However, circulating influenza viruses in humans are subject to permanent antigenic changes which require annual adaptation of the influenza vaccine formulation to ensure the closest possible match between the influenza vaccine strains and the circulating influenza strains. Although yearly vaccination with influenza vaccines is the best way to prevent influenza, antiviral drugs, such as oseltamivir (Tamiflu®) can be effective for prevention and treatment of influenza infection. The number of influenza virus strains showing resistance against antiviral drugs, such as oseltamivir is, however, increasing.

An alternative approach is the development of antibody-based prophylactic or therapeutic treatments to neutralize various seasonal and pandemic influenza viruses. The primary target of most neutralizing antibodies that protect against influenza virus infection is the globular head (HA1 part) of the viral HA protein which contains the receptor binding site, but which is subject to continuing genetic evolution with amino acid substitutions in antibody-binding sites (antigenic drift).

Recently, broadly cross-neutralizing antibodies recognizing an epitope in the conserved stem region of hemagglutinin of influenza A viruses of phylogenetic group 1 (including e.g. the H1 and H5 influenza subtypes) have been identified (see e.g. WO2008/028946), as well as cross-neutralizing antibodies recognizing a highly conserved epitope in the stem region of HA of influenza A viruses of phylogenetic group 2 (including e.g. H3 and H7 subtypes) (WO 2010/130636). The neutralizing activity of these antibodies is restricted to either group 1 or group 2 influenza viruses. In addition, these antibodies are not capable of binding to and neutralizing influenza B viruses.

Furthermore, WO 2010/010466 discloses a human antibody FI6 binding to hemagglutinin and capable of binding to and neutralizing influenza A subtypes of group 1 (including H1 and H5 subtypes) and group 2 (including H3 and H7 subtypes). This antibody also does not bind HA from influenza B viruses.

In addition, US 2009/0092620 discloses a murine antibody recognizing an antigenic structure present in hemagglutinin of both the H1 and the H3 subtype and on hemagglutinin of influenza B viruses belonging to the B/Victoria and B/Yamagata groups. The antibodies inhibit the hemagglutination activity of several H3N2 strains implicating that this antibody binds an epitope in the globular head of HA.

In view of the severity of the respiratory illness caused by influenza A and influenza B viruses, as well has the high economic impact of the seasonal epidemics, and the continuing risk for pandemics, there is an ongoing need for effective means for the prevention and treatment of influenza A and B subtypes. There is thus a need for binding molecules, preferably broadly neutralizing human binding molecules, capable of cross-neutralizing influenza A viruses of both phylogenetic group 1 and phylogenetic group 2, and preferably also influenza B viruses.

SUMMARY OF THE INVENTION

The invention provides binding molecules capable of specifically binding to influenza A virus strains from both phylogenetic group 1 (including e.g. influenza viruses comprising HA of the H1 and H5 subtype) and influenza A virus strains from phylogenetic group 2 (including e.g. influenza viruses comprising HA of the H3 and H7 subtype). In an embodiment, the binding molecules also have neutralizing activity against

influenza A virus strains from both phylogenetic group 1 and phylogenetic group 2. In an embodiment, the binding molecules are furthermore capable of specifically binding influenza B virus strains, including e.g. influenza B virus strains of the B/Yamagata and/or B/Victoria lineages. In an embodiment, the binding molecules are furthermore capable of neutralizing influenza B virus strains, including e.g. influenza B virus strains of the B/Yamagata and/or B/Victoria lineages. In an embodiment, the binding molecules are capable of in vivo neutralizing influenza A and/or B virus strains. In an embodiment the binding molecules bind to a conserved epitope in the stem region of the HA protein of influenza A and B viruses. In an embodiment, the binding molecules have no hemagglutination inhibiting (HI) activity.

The invention thus provides binding molecules that bind to an epitope in the stem region of the haemagglutinin protein that is shared between influenza A virus subtypes within the phylogenetic group 1 and influenza virus subtypes within phylogenetic group 2, as well as influenza B virus subtypes, and therefore relates to binding molecules that cross-react between both group 1 and group 2 influenza A virus subtypes and influenza B viruses. The invention also pertains to nucleic acid molecules encoding at least the binding region of the human binding molecules.

The binding molecules and/or nucleic acid molecules of the invention are suitable for use as a universal prophylactic, diagnostic and/or treatment agent for influenza A viruses and influenza B viruses, even irrespective of the causative influenza subtype.

It is surmised that the binding molecules according to the present invention bind to hitherto unknown and highly conserved epitopes that are not or much less prone to antigenic drift or shift. In particular, this epitope is shared between influenza viruses belonging to both phylogenetic group 1 and phylogenetic group 2, and influenza B viruses. It is also encompassed to use the binding molecules of the invention to identify and/or characterize these epitopes.

The invention further provides the use of the human binding molecules and/or the nucleic acid molecules of the invention in the diagnosis, prophylaxis and/or treatment of a subject having, or at risk of developing, an influenza virus infection. Furthermore, the invention pertains to the use of the human binding molecules and/or the nucleic acid molecules of the invention in the diagnosis/detection of such influenza infections.

DESCRIPTION OF THE FIGURES

FIG. 1 shows the blocking of conformational change of H1, H5, H9, H3, and H7 HAs by CR9114. (**A**) FACS binding of CR9114 to various conformations – uncleaved precursor (HA0); neutral pH, cleaved (HA); fusion pH, cleaved (fusion pH) – of surface-expressed rHA of A/New Caledonia/20/1999 (H1) A/Viet Nam/1203/2004 (H5), A/Hong Kong/1073/1999 (H9), A/Wisconsin/67/2005 (H3), and A/Netherlands/219/2003 (H7). Binding is expressed as the percentage of binding to untreated rHA (HA0). (**B**) FACS binding of CR9114 to surface-expressed HA as above, except that mAb CR9114 was added before exposure of the cleaved HAs to a pH of 4.9.

FIG. 2 shows that MAb CR9114 competes with CR6261 and CR8020 for binding to H1 and H3, respectively. Additional degree of binding of indicated mAbs to immobilized HA of A/New Caledonia/20/1999 (H1N1) saturated with 100 nM of CR6261 or CR9114 (panels **A** and **B**), or to immobilized HA of A/Wisconsin/67/2005 (H3N2) saturated with 100 nM of CR8020 or CR9114 (panels **C** and **D**), measured using biolayer interferometry.

FIG. 3 demonstrates the prophylactic efficacy of CR9114 in the mouse lethal challenge model with influenza B (B/Florida/04/2006) virus. **A**. Kaplan-Meier survival curves of mice treated intravenously with either 15 mg/kg CR9114 or vehicle control on day -1 before challenge, followed by a challenge at day 0 of 25 LD B/Florida/04/2006. **B**. Mean bodyweight change (%) relative to day 0. Bars represent 95% CI of the mean. If a mouse died/was euthanized during the study, the last observed bodyweight was carried forward. **C**. Median Clinical scores. Bars represent interquartile ranges. Clinical score explanation: 0=no clinical signs; 1=rough coat; 2=rough coat, less reactive during handling; 3=rough coat, rolled up, laboured breathing, less reactive during handling; 4=rough coat, rolled up, laboured breathing, inactive response to manipulation/handlings.

DESCRIPTION OF THE INVENTION

Definitions of terms as used in the present invention are given below.

The term “included” or “including” as used herein is deemed to be followed by the words “without limitation”.

As used herein the term "binding molecule" refers to an intact immunoglobulin including monoclonal antibodies, such as chimeric, humanized or human monoclonal antibodies, or to an antigen-binding and/or variable domain comprising fragment of an immunoglobulin that competes with the intact immunoglobulin for specific binding to the binding partner of the immunoglobulin, e.g. HA. Regardless of structure, the antigen-binding fragment binds with the same antigen that is recognized by the intact immunoglobulin. An antigen-binding fragment can comprise a peptide or polypeptide comprising an amino acid sequence of at least 2, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, or 250 contiguous amino acid residues of the amino acid sequence of the binding molecule.

The term "binding molecule", as used herein includes all immunoglobulin classes and subclasses known in the art. Depending on the amino acid sequence of the constant domain of their heavy chains, binding molecules can be divided into the five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgA1, IgA2, IgG1, IgG2, IgG3 and IgG4.

Antigen-binding fragments include, inter alia, Fab, F(ab'), F(ab')2, Fv, dAb, Fd, complementarity determining region (CDR) fragments, single-chain antibodies (scFv), bivalent single-chain antibodies, single-chain phage antibodies, diabodies, triabodies, tetrabodies, (poly)peptides that contain at least a fragment of an immunoglobulin that is sufficient to confer specific antigen binding to the (poly)peptide, etc. The above fragments may be produced synthetically or by enzymatic or chemical cleavage of intact immunoglobulins or they may be genetically engineered by recombinant DNA techniques. The methods of production are well known in the art and are described, for example, in Antibodies: A Laboratory Manual, Edited by: E. Harlow and D, Lane (1988), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, which is incorporated herein by reference. A binding molecule or antigen-binding fragment thereof may have one or more binding sites. If there is more than one binding site, the binding sites may be identical to one another or they may be different.

The binding molecule can be a naked or unconjugated binding molecule but can also be part of an immunoconjugate. A naked or unconjugated binding molecule is intended to refer to a binding molecule that is not conjugated, operatively linked or

otherwise physically or functionally associated with an effector moiety or tag, such as inter alia a toxic substance, a radioactive substance, a liposome, an enzyme. It will be understood that naked or unconjugated binding molecules do not exclude binding molecules that have been stabilized, multimerized, humanized or in any other way manipulated, other than by the attachment of an effector moiety or tag. Accordingly, all post-translationally modified naked and unconjugated binding molecules are included herewith, including where the modifications are made in the natural binding molecule-producing cell environment, by a recombinant binding molecule-producing cell, and are introduced by the hand of man after initial binding molecule preparation. Of course, the term naked or unconjugated binding molecule does not exclude the ability of the binding molecule to form functional associations with effector cells and/or molecules after administration to the body, as some of such interactions are necessary in order to exert a biological effect. The lack of associated effector group or tag is therefore applied in definition to the naked or unconjugated binding molecule in vitro, not in vivo.

As used herein, the term "biological sample" encompasses a variety of sample types, including blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures, or cells derived there from and the progeny thereof. The term also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term encompasses various kinds of clinical samples obtained from any species, and also includes cells in culture, cell supernatants and cell lysates.

The term "complementarity determining regions" (CDR) as used herein means sequences within the variable regions of binding molecules, such as immunoglobulins, that usually contribute to a large extent to the antigen binding site which is complementary in shape and charge distribution to the epitope recognized on the antigen. The CDR regions can be specific for linear epitopes, discontinuous epitopes, or conformational epitopes of proteins or protein fragments, either as present on the protein in its native conformation or, in some cases, as present on the proteins as denatured, e.g., by solubilization in SDS. Epitopes may also consist of posttranslational modifications of proteins.

The term "deletion", as used herein, denotes a change in either amino acid or nucleotide sequence in which one or more amino acid or nucleotide residues, respectively, are absent as compared to the reference, often the naturally occurring, molecule.

The term "expression-regulating nucleic acid sequence" as used herein refers to polynucleotide sequences necessary for and/or affecting the expression of an operably linked coding sequence in a particular host organism. The expression-regulating nucleic acid sequences, such as inter alia appropriate transcription initiation, termination, promoter, enhancer sequences; repressor or activator sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (e.g., ribosome binding sites); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion, can be any nucleic acid sequence showing activity in the host organism of choice and can be derived from genes encoding proteins, which are either homologous or heterologous to the host organism. The identification and employment of expression-regulating sequences is routine to the person skilled in the art.

The term "functional variant", as used herein, refers to a binding molecule that comprises a nucleotide and/or amino acid sequence that is altered by one or more nucleotides and/or amino acids compared to the nucleotide and/or amino acid sequences of the reference binding molecule and that is capable of competing for binding to the binding partner, i.e. the influenza virus, with the reference binding molecule. In other words, the modifications in the amino acid and/or nucleotide sequence of the reference binding molecule do not significantly affect or alter the binding characteristics of the binding molecule encoded by the nucleotide sequence or containing the amino acid sequence, i.e. the binding molecule is still able to recognize and bind its target. The functional variant may have conservative sequence modifications including nucleotide and amino acid substitutions, additions and deletions. These modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and random PCR-mediated mutagenesis, and may comprise natural as well as non-natural nucleotides and amino acids.

Conservative amino acid substitutions include the ones in which the amino acid residue is replaced with an amino acid residue having similar structural or chemical properties. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), non-polar side chains (e.g., glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan). It will be clear to the skilled artisan that also other classifications of amino acid residue families than the one used above can be employed. Furthermore, a variant may have non-conservative amino acid substitutions, e.g., replacement of an amino acid with an amino acid residue having different structural or chemical properties. Similar minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing immunological activity may be found using computer programs well known in the art.

A mutation in a nucleotide sequence can be a single alteration made at a locus (a point mutation), such as transition or transversion mutations, or alternatively, multiple nucleotides may be inserted, deleted or changed at a single locus. In addition, one or more alterations may be made at any number of loci within a nucleotide sequence. The mutations may be performed by any suitable method known in the art.

The term “influenza virus subtype” as used herein in relation to influenza A viruses refers to influenza A virus variants that are characterized by various combinations of the hemagglutinin (H) and neuramidase (N) viral surface proteins. According to the present invention influenza virus subtypes may be referred to by their H number, such as for example “influenza virus comprising HA of the H1 or H3 subtype”, or “H1 influenza virus” “H3 influenza virus”, or by a combination of a H number and an N number, such as for example “influenza virus subtype H3N2” or “H3N2”.

The term influenza virus “subtype” specifically includes all individual influenza virus “strains” within each subtype, which usually result from mutations and show different pathogenic profiles. Such strains may also be referred to as various “isolates” of

a viral subtype. Accordingly, as used herein, the terms "strains" and "isolates" may be used interchangeably. The current nomenclature for human influenza virus strains or isolates includes the geographical location of the first isolation, strain number and year of isolation, usually with the antigenic description of HA and NA given in brackets, e.g. A/Moscow/10/00 (H3N2). Non-human strains also include the host of origin in the nomenclature.

The term "neutralizing" as used herein in relation to the binding molecules of the invention refers to binding molecules that inhibit an influenza virus from replicatively infecting a target cell, regardless of the mechanism by which neutralization is achieved. Thus, neutralization can e.g. be achieved by inhibiting the attachment or adhesion of the virus to the cell surface, or by inhibition of the fusion of viral and cellular membranes following attachment of the virus to the target cell, and the like.

The term "cross-neutralizing" or "cross-neutralization" as used herein in relation to the binding molecules of the invention refers to the ability of the binding molecules of the invention to neutralize different subtypes of influenza A and/or B viruses.

The term "host", as used herein, is intended to refer to an organism or a cell into which a vector such as a cloning vector or an expression vector has been introduced. The organism or cell can be prokaryotic or eukaryotic. Preferably, the hosts isolated host cells, e.g. host cells in culture. The term "host cells" merely signifies that the cells are modified for the (over)-expression of the binding molecules of the invention and include B-cells that originally express these binding molecules and which cells have been modified to over-express the binding molecule by immortalization, amplification, enhancement of expression etc. It should be understood that the term host is intended to refer not only to the particular subject organism or cell but to the progeny of such an organism or cell as well. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent organism or cell, but are still included within the scope of the term "host" as used herein.

The term "human", when applied to binding molecules as defined herein, refers to molecules that are either directly derived from a human or based upon a human germ line sequence. When a binding molecule is derived from or based on a human sequence and

subsequently modified, it is still to be considered human as used throughout the specification. In other words, the term human, when applied to binding molecules is intended to include binding molecules having variable and constant regions derived from human germline immunoglobulin sequences or based on variable or constant regions occurring in a human or human lymphocyte and modified in some form. Thus, the human binding molecules may include amino acid residues not encoded by human germline immunoglobulin sequences, comprise substitutions and/or deletions (*e.g.*, mutations introduced by for instance random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). "Based on" as used herein refers to the situation that a nucleic acid sequence may be exactly copied from a template, or with minor mutations, such as by error-prone PCR methods, or synthetically made matching the template exactly or with minor modifications.

The term "insertion", also known as the term "addition", denotes a change in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid or nucleotide residues, respectively, as compared to the parent sequence.

The term "isolated", when applied to binding molecules as defined herein, refers to binding molecules that are substantially free of other proteins or polypeptides, particularly free of other binding molecules having different antigenic specificities, and are also substantially free of other cellular material and/or chemicals. For example, when the binding molecules are recombinantly produced, they are preferably substantially free of culture medium components, and when the binding molecules are produced by chemical synthesis, they are preferably substantially free of chemical precursors or other chemicals, *i.e.*, they are separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. The term "isolated" when applied to nucleic acid molecules encoding binding molecules as defined herein, is intended to refer to nucleic acid molecules in which the nucleotide sequences encoding the binding molecules are free of other nucleotide sequences, particularly nucleotide sequences encoding binding molecules that bind other binding partners. Furthermore, the term "isolated" refers to nucleic acid molecules that are substantially separated from other cellular components that naturally accompany the native nucleic acid molecule in its natural host, *e.g.*, ribosomes, polymerases, or genomic sequences with which it is naturally associated.

Moreover, "isolated" nucleic acid molecules, such as cDNA molecules, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

The term "monoclonal antibody" as used herein refers to a preparation of antibody molecules of single specificity. A monoclonal antibody displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to an antibody displaying a single binding specificity which has variable and constant regions derived from or based on human germline immunoglobulin sequences or derived from completely synthetic sequences. The method of preparing the monoclonal antibody is not relevant for the binding specificity.

The term "naturally occurring" as used herein as applied to an object refers to the fact that an object or compound can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring.

The term "nucleic acid molecule" as used in the present invention refers to a polymeric form of nucleotides and includes both sense and anti-sense strands of RNA, cDNA, genomic DNA, and synthetic forms and mixed polymers of the above. A nucleotide refers to a ribonucleotide, deoxynucleotide or a modified form of either type of nucleotide. The term also includes single- and double-stranded forms of DNA. In addition, a polynucleotide may include either or both naturally occurring and modified nucleotides linked together by naturally occurring and/or non-naturally occurring nucleotide linkages. The nucleic acid molecules may be modified chemically or biochemically or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those of skill in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analogue, internucleotide modifications such as uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, etc.), pendent moieties (*e.g.*, polypeptides), intercalators (*e.g.*, acridine, psoralen, etc.), chelators, alkylators, and modified linkages

(*e.g.*, alpha anomeric nucleic acids, etc.). The above term is also intended to include any topological conformation, including single-stranded, double-stranded, partially duplexed, triplex, hairpinned, circular and padlocked conformations. Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule. A reference to a nucleic acid sequence encompasses its complement unless otherwise specified. Thus, a reference to a nucleic acid molecule having a particular sequence should be understood to encompass its complementary strand, with its complementary sequence. The complementary strand is also useful, *e.g.*, for anti-sense therapy, hybridisation probes and PCR primers.

The term "operably linked" refers to two or more nucleic acid sequence elements that are usually physically linked and are in a functional relationship with each other. For instance, a promoter is operably linked to a coding sequence, if the promoter is able to initiate or regulate the transcription or expression of a coding sequence, in which case the coding sequence should be understood as being "under the control of" the promoter.

By "pharmaceutically acceptable excipient" is meant any inert substance that is combined with an active molecule such as a drug, agent, or binding molecule for preparing an agreeable or convenient dosage form. The "pharmaceutically acceptable excipient" is an excipient that is non-toxic to recipients at the used dosages and concentrations, and is compatible with other ingredients of the formulation comprising the drug, agent or binding molecule. Pharmaceutically acceptable excipients are widely applied and known in the art.

The term "specifically binding", as used herein, in reference to the interaction of a binding molecule, *e.g.* an antibody, and its binding partner, *e.g.* an antigen, means that the interaction is dependent upon the presence of a particular structure, *e.g.* an antigenic determinant or epitope, on the binding partner. In other words, the antibody preferentially binds or recognizes the binding partner even when the binding partner is present in a mixture of other molecules or organisms. The binding may be mediated by covalent or non-covalent interactions or a combination of both. In yet other words, the term "specifically binding" means immunospecifically binding to an antigenic determinant or

epitope and not immunospecifically binding to other antigenic determinants or epitopes. A binding molecule that immunospecifically binds to an antigen may bind to other peptides or polypeptides with lower affinity as determined by, *e.g.*, radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), BIACORE, or other assays known in the art. Binding molecules or fragments thereof that immunospecifically bind to an antigen may be cross-reactive with related antigens, carrying the same epitope. Preferably, binding molecules or fragments thereof that immunospecifically bind to an antigen do not cross-react with other antigens.

A "substitution", as used herein, denotes the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

The term "therapeutically effective amount" refers to an amount of the binding molecule as defined herein that is effective for preventing, ameliorating and/or treating a condition resulting from infection with an influenza B virus. Ameloriation as used in herein may refer to the reduction of visible or perceptible disease symptoms, viremia, or any other measurable manifestation of influenza infection.

The term "treatment" refers to therapeutic treatment as well as prophylactic or preventative measures to cure or halt or at least retard disease progress. Those in need of treatment include those already inflicted with a condition resulting from infection with influenza virus as well as those in which infection with influenza virus is to be prevented. Subjects partially or totally recovered from infection with influenza virus might also be in need of treatment. Prevention encompasses inhibiting or reducing the spread of influenza virus or inhibiting or reducing the onset, development or progression of one or more of the symptoms associated with infection with influenza virus.

The term "vector" denotes a nucleic acid molecule into which a second nucleic acid molecule can be inserted for introduction into a host where it will be replicated, and in some cases expressed. In other words, a vector is capable of transporting a nucleic acid molecule to which it has been linked. Cloning as well as expression vectors are contemplated by the term "vector", as used herein. Vectors include, but are not limited to, plasmids, cosmids, bacterial artificial chromosomes (BAC) and yeast artificial chromosomes (YAC) and vectors derived from bacteriophages or plant or animal (including human) viruses. Vectors comprise an origin of replication recognized by the

proposed host and in case of expression vectors, promoter and other regulatory regions recognized by the host. A vector containing a second nucleic acid molecule is introduced into a cell by transformation, transfection, or by making use of viral entry mechanisms. Certain vectors are capable of autonomous replication in a host into which they are introduced (*e.g.*, vectors having a bacterial origin of replication can replicate in bacteria). Other vectors can be integrated into the genome of a host upon introduction into the host, and thereby are replicated along with the host genome.

DETAILED DESCRIPTION

In a first aspect the present invention encompasses binding molecules capable of specifically binding to hemagglutinin (HA) of influenza A virus subtypes of phylogenetic group 1 and influenza A virus subtypes of phylogenetic group 2. In an embodiment, the binding molecules are capable of neutralizing influenza A virus subtypes of both phylogenetic group 1 and phylogenetic group 2. The binding molecules of the invention thus are unique in that they are capable of cross-neutralizing group 1 influenza A virus strains and group 2 influenza A virus strains. In an embodiment, the binding molecules are capable of neutralizing at least one or more, preferably two or more, preferably three or more, preferably four or more, even more preferably five or more group 1 influenza A virus subtypes selected from the group consisting of the H1, H2, H5, H6, H8, H9 and H11 subtype, and at least one or more, preferably two or more, preferably three or more group 2 influenza A virus subtypes selected from the group consisting of the H3, H4, H7, and H10 subtype. In an embodiment, the binding molecules are capable of specifically binding to hemagglutinin (HA) of influenza B virus subtypes. In another embodiment, the binding molecules are capable of neutralizing influenza B viruses. In an embodiment, the binding molecules are capable of *in vivo* neutralizing influenza A and/or B viruses. The influenza A and B virus strains may be both human and non-human influenza virus strains (i.e. obtained from non-human animals, e.g. birds).

Preferably, the binding molecules are human binding molecules. In a preferred embodiment, the binding molecules are human antibodies, or antigen-binding fragments thereof.

In an embodiment, the binding molecules are derived from the VH1-69 germ line gene. Thus, the binding molecules all use the same VH1-69 germ line encoded framework.

In an embodiment, the binding interaction of the binding molecules, preferably the antibody, and HA is mediated exclusively by heavy chain variable sequences.

In an embodiment, the binding molecules comprise a heavy chain CDR1 comprising the amino acid sequence of SEQ ID NO: 133 or SEQ ID NO: 139, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 134, SEQ ID NO: 140 or SEQ ID NO: 151, and a heavy chain CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 135, SEQ ID NO: 141, SEQ ID NO: 145, SEQ ID NO: 152, SEQ ID NO: 161, and SEQ ID NO: 162. The CDR regions of binding molecules of the invention are shown in Table 7. CDR regions are according to Kabat *et al.* (1991) as described in Sequences of Proteins of Immunological Interest.

Influenza viruses infect cells by binding to sialic acid residues on the cell surface of target cells, and following transfer into endosomes, by fusing their membranes with the endosomal membranes and releasing the genome-transcriptase complex into the cell. Both receptor binding and membrane fusion process are mediated by the HA glycoprotein. The HA of influenza virus A comprises two structurally distinct regions, i.e. a globular head region, which contains a receptor binding site which is responsible for virus attachment to the target cell, and is involved in the haemagglutination activity of HA, and a stem region, containing a fusion peptide which is necessary for membrane fusion between the viral envelope and the endosomal membrane of the cell. The HA protein is a trimer in which each monomer consists of two disulphide - linked glycopolypeptides, HA1 and HA2, that are produced during infection by proteolytic cleavage of a precursor (HA0). Cleavage is necessary for virus infectivity since it is required to prime the HA for membrane fusion, to allow conformational change. Activation of the primed molecule occurs at low pH in endosomes, between pH5 and pH6, and requires extensive changes in HA structure. Each of the stages in the priming and activation of HA for its participation in the membrane fusion process, presents a different target for inhibition, e.g. by monoclonal antibodies. In an embodiment, the

binding molecules are capable of blocking the pH-induced conformational changes in HA associated with membrane fusion.

The binding molecules of the invention may be capable of specifically binding to the HA0, HA1 and/or HA2 subunit of the HA protein. They may be capable of specifically binding to linear or structural and/or conformational epitopes on the HA0, HA1 and/or HA2 subunit of the HA protein. The HA molecule may be purified from viruses or recombinantly produced and optionally isolated before use. Alternatively, HA may be expressed on the surface of cells. In an embodiment, the binding molecules of the invention are capable of specifically binding to an epitope in the stem region of HA. In an embodiment, the binding molecules bind to an epitope that is accessible in the pre-fusion conformation of HA.

The binding molecules of the invention may be capable of specifically binding to influenza viruses that are viable, living and/or infective or that are in inactivated/attenuated form. Methods for inactivating/attenuating virus, e.g. influenza viruses are well known in the art and include, but are not limited to, treatment with formalin, β-propiolactone (BPL), merthiolate, and/or ultraviolet light.

The binding molecules of the invention may also be capable of specifically binding to one or more fragments of the influenza viruses, such as *inter alia* a preparation of one or more proteins and/or (poly)peptides, derived from subtypes of influenza A and/or B viruses or one or more recombinantly produced proteins and/or polypeptides of influenza A and/or B viruses. The nucleotide and/or amino acid sequence of proteins of various influenza A and B strains can be found in the GenBank-database, NCBI Influenza Virus Sequence Database, Influenza Sequence Database (ISD), EMBL-database and/or other databases. It is well within the reach of the skilled person to find such sequences in the respective databases.

In another embodiment the binding molecules of the invention are capable of specifically binding to a fragment of the above-mentioned proteins and/or polypeptides, wherein the fragment at least comprises an epitope recognized by the binding molecules of the invention. An "epitope" as used herein is a moiety that is capable of binding to a binding molecule of the invention with sufficiently high affinity to form a detectable antigen-binding molecule complex.

The binding molecules of the invention may or may not be capable of specifically binding to the extracellular part of HA (also called herein soluble HA (sHA)).

The binding molecules of the invention can be intact immunoglobulin molecules such as polyclonal or monoclonal antibodies or the binding molecules can be antigen-binding fragments thereof, including, but not limited to, heavy and light chain variable regions, Fab, F(ab'), $F(ab')_2$, Fv, dAb, Fd, complementarity determining region (CDR) fragments, single-chain antibodies (scFv), bivalent single-chain antibodies, single-chain phage antibodies, diabodies, triabodies, tetrabodies, and (poly)peptides that contain at least a fragment of an immunoglobulin that is sufficient to confer specific antigen binding to influenza virus strains or a fragment thereof. In a preferred embodiment the binding molecules of the invention are human monoclonal antibodies, and/or antigen-binding fragments thereof. The binding molecules may also be Nanobodies, alphabodies, affibodies, FN3-domain scaffolds and other scaffolds based on domains in (human) repeat proteins like Adnectins, Anticalins, Darpins, etc, or other scaffolds comprising epitope binding sequences.

The binding molecules of the invention can be used in non-isolated or isolated form. Furthermore, the binding molecules of the invention can be used alone or in a mixture comprising at least one binding molecule (or variant or fragment thereof) of the invention, and/or with other binding molecules that bind to influenza and have influenza virus inhibiting effect. In other words, the binding molecules can be used in combination, *e.g.*, as a pharmaceutical composition comprising two or more binding molecules of the invention, variants or fragments thereof. For example, binding molecules having different, but complementary activities can be combined in a single therapy to achieve a desired prophylactic, therapeutic or diagnostic effect, but alternatively, binding molecules having identical activities can also be combined in a single therapy to achieve a desired prophylactic, therapeutic or diagnostic effect. Optionally, the mixture further comprises at least one other therapeutic agent. Preferably, the therapeutic agent such as, *e.g.*, M2 inhibitors (*e.g.*, amantidine, rimantadine) and/or neuraminidase inhibitors (*e.g.*, zanamivir, oseltamivir) is useful in the prophylaxis and/or treatment of an influenza virus infection

Typically, binding molecules according to the invention can bind to their binding partners, i.e. an influenza A virus of group 1 (such as H1N1) and an influenza A virus of group 2 (such as H3N2), and/or an influenza B virus, and/or fragments thereof, with an affinity constant (K_d-value) that is lower than $0.2x10^{-4}$ M, $1.0x10^{-5}$ M, $1.0x10^{-6}$ M, $1.0x10^{-7}$ M, preferably lower than $1.0x10^{-8}$ M, more preferably lower than $1.0x10^{-9}$ M, more preferably lower than $1.0x10^{-10}$ M, even more preferably lower than $1.0x10^{-11}$ M, and in particular lower than $1.0x10^{-12}$ M. The affinity constants can vary for antibody isotypes. For example, affinity binding for an IgM isotype refers to a binding affinity of at least about $1.0x10^{-7}$ M. Affinity constants can for instance be measured using surface plasmon resonance, for example using the BIACORE system (Pharmacia Biosensor AB, Uppsala, Sweden).

The binding molecules of the invention exhibit neutralizing activity. Neutralizing activity can for instance be measured as described herein. Alternative assays measuring neutralizing activity are described in for instance WHO Manual on Animal Influenza Diagnosis and Surveillance, Geneva: World Health Organisation, 2005, version 2002.5.

Typically, the binding molecules according to the invention have a neutralizing activity of 50µg/ml or less, preferably 20µg/ml or less, more preferably a neutralizing activity of 10 µg/ml or less, even more preferably 5 µg/ml or less, as determined in an *in vitro* virus neutralization assay (VNA) as described in Example 6. The binding molecules according to the invention may bind to influenza virus or a fragment thereof in soluble form such as for instance in a sample or in suspension or may bind to influenza viruses or fragments thereof bound or attached to a carrier or substrate, *e.g.*, microtiter plates, membranes and beads, etc. Carriers or substrates may be made of glass, plastic (*e.g.*, polystyrene), polysaccharides, nylon, nitrocellulose, or Teflon, etc. The surface of such supports may be solid or porous and of any convenient shape. Furthermore, the binding molecules may bind to influenza virus in purified/isolated or non-purified/non-isolated form.

As discussed above, the present invention relates to isolated human binding molecules that are able to recognize and bind to an epitope in the influenza haemagglutinin protein (HA) wherein said binding molecules have neutralizing activity against influenza A viruses of phylogenetic group 1 and influenza A viruses of

phylogenetic group 2. According to the invention, it thus has been shown that the binding molecules of the present invention cross-neutralize influenza virus subtypes belonging to both phylogenetic groups. The skilled person, based on what has been disclosed herein, can determine whether an antibody indeed cross-reacts with HA proteins from different subtypes and can also determine whether they are able to neutralize influenza viruses of different subtypes in vitro and/or *in vivo*.

In an embodiment the binding molecule according to the present invention is selected from the group consisting of:

a) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:133, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:135,

b) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:140, and a heavy chain CDR3 region of SEQ ID NO:141,

c) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:145,

d) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:151, and a heavy chain CDR3 region of SEQ ID NO:152,

e) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152,

f) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:151, and a heavy chain CDR3 region of SEQ ID NO:161,

g) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:151, and a heavy chain CDR3 region of SEQ ID NO:162, and

h) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:141.

In a preferred embodiment, the binding molecule comprises a heavy chain CDR1 region comprising the amino acid sequence of SEQ ID NO:139, a heavy chain CDR2 region comprising an amino acid sequence of SEQ ID NO:134, and a heavy chain CDR3 region comprising the amino acid sequence of SEQ ID NO:145 or SEQ ID NO: 152.

In another embodiment, the human binding molecules according to the invention are selected from the group consisting of:

a) a binding molecule having a heavy chain CDR1 region of SEQ ID NO:133, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:135, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:136, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:137, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:138,

b) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:140, and a heavy chain CDR3 region of SEQ ID NO:141, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:142, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:143, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:144,

c) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:145, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:146, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:174, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:147,

d) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:145, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:148, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:149, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:150,

e) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:151, and a heavy chain CDR3 region of SEQ

ID NO:152, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:153, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:154, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:155,

f) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:148, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:149, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:150,

g) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:156, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:157, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:158,

h) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:148, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:159, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:160,

i) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:151, and a heavy chain CDR3 region of SEQ ID NO:161, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:142, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:143, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:144,

j) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:151, and a heavy chain CDR3 region of SEQ ID NO:162, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:163, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:164, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:165,

k) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, a light chain CDR1 region having the amino acid sequence of SEQ ID

NO:166, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:167, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:168,

l) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:169, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:149, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:150,

m) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:141, a light chain CDR1 region having the amino acid sequence of SEQ ID NO: 163, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:169, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:170,

n) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:171, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:164, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:172,

o) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:145, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:142, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:143, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:173, and

p) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:142, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:143, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:144.

In another embodiment, the human binding molecules according to the invention are selected from the group consisting of:

a) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ

ID NO:145, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:146, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:174, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:147,

b) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:171, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:164, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:172,

c) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:145, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:142, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:143, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:173, and

d) a binding molecule comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, a light chain CDR1 region having the amino acid sequence of SEQ ID NO:142, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:143, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:144.

In another embodiment, the binding molecule according to the invention is selected from the group consisting of

a) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 2,
b) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 6,
c) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 10,
d) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 14,
e) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 18,
f) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 22,
g) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 26,
h) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 30,
i) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 34,
j) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 38,
k) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 42,

l) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 46,

m) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 50,

n) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 54,

o) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 58, and

p) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 62.

In an embodiment, the binding molecule according to the invention is selected from the group consisting of a binding molecule comprising a heavy chain variable region of SEQ ID NO: 10 a binding molecule comprising a heavy chain variable region of SEQ ID NO: 54, a binding molecule comprising a heavy chain variable region of SEQ ID NO: 58, and a binding molecule comprising a heavy chain variable region of SEQ ID NO: 62.

In a further embodiment, the binding molecules according to the invention comprise a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:20, SEQ ID NO:24, SEQ ID NO:28, SEQ ID NO:32, SEQ ID NO:36, SEQ ID NO:40, SEQ ID NO:44, SEQ ID NO:48, SEQ ID NO:52, SEQ ID NO:56, SEQ ID NO:60, and SEQ ID NO:64.

In yet another embodiment, the binding molecule is selected from the group consisting of

a) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 2 and a light chain variable region of SEQ ID NO: 4,

b) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 6 and a light chain variable region of SEQ ID NO: 8,

c) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 10 and a light chain variable region of SEQ ID NO: 12,

d) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 14 and a light chain variable region of SEQ ID NO: 16,

e) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 18 and a light chain variable region of SEQ ID NO: 20,

f) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 22 and a light chain variable region of SEQ ID NO: 24,

g) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 26 and a light chain variable region of SEQ ID NO: 28,

h) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 30 and a light chain variable region of SEQ ID NO: 32,

i) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 34 and a light chain variable region of SEQ ID NO: 36,

j) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 38 and a light chain variable region of SEQ ID NO: 40,

k) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 42 and a light chain variable region of SEQ ID NO: 44,

l) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 46 and a light chain variable region of SEQ ID NO: 48,

m) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 50 and a light chain variable region of SEQ ID NO: 52,

n) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 54 and a light chain variable region of SEQ ID NO: 56,

o) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 58 and a light chain variable region of SEQ ID NO: 60, and

p) a binding molecule comprising a heavy chain variable region of SEQ ID NO: 62 and a light chain variable region of SEQ ID NO: 64.

In an embodiment, the human binding molecules according to the invention are selected from the group consisting of: a binding molecule comprising a heavy chain variable region of SEQ ID NO: 10 and a light chain variable region of SEQ ID NO: 12, a binding molecule comprising a heavy chain variable region of SEQ ID NO: 54 and a light chain variable region of SEQ ID NO: 56, a binding molecule comprising a heavy chain variable region of SEQ ID NO: 58 and a light chain variable region of SEQ ID NO: 60, and a binding molecule comprising a heavy chain variable region of SEQ ID NO: 62 and a light chain variable region of SEQ ID NO: 64.

In a preferred embodiment, the binding molecules according to the invention are for a use as a medicament, and preferably for use in the diagnostic, therapeutic and/or prophylactic treatment of influenza infection caused by influenza A and/or B viruses.

Preferably, the influenza virus that causes the influenza infection and that can be treated using the binding molecules of the present invention is an influenza A virus of phylogenetic group 1 and/or 2, and/or a influenza B virus. The present invention also relates to a pharmaceutical composition comprising at least one binding molecule according to the invention, and a pharmaceutically acceptable excipient.

In yet another embodiment the invention relates to the use of a binding molecule according to the invention in the preparation of a medicament for the diagnosis, prophylaxis, and/or treatment of an influenza virus infection. Such infections can occur in small populations, but can also spread around the world in seasonal epidemics or, worse, in global pandemics where millions of individuals are at risk. The invention provides binding molecules that can neutralize the infection of influenza strains that cause such seasonal epidemics, as well as potential pandemics. Importantly, protection and treatment can be envisioned now with the binding molecules of the present invention in relation to various influenza subtypes as it has been disclosed that the binding molecules of the present invention are capable of cross-neutralizing various influenza subtypes of both phylogenetic group 1, encompassing H1, H2, H5, H6, H8, H9 and H11 subtypes and phylogenetic group 2, encompassing subtypes H3, H4, H7 and H10 subtypes, as well as influenza B subtypes.

Another aspect of the invention includes functional variants of the binding molecules as defined herein. Molecules are considered to be functional variants of a binding molecule according to the invention, if the variants are capable of competing for specifically binding to an influenza virus or a fragment thereof with the “parental” or “reference” binding molecules. In other words, molecules are considered to be functional variants of a binding molecule according to the invention when the functional variants are still capable of binding to the same or overlapping epitope of the influenza virus or a fragment thereof. For the sake of this application “parental” and “reference” will be used as synonyms meaning that the information of the reference or parental molecule, or the physical molecule itself may form the basis for the variation. Functional variants include, but are not limited to, derivatives that are substantially similar in primary structural sequence, including those that have modifications in the Fc receptor or other regions involved with effector functions, and/or which contain *e.g. in vitro* or *in vivo*

modifications, chemical and/or biochemical, that are not found in the parental binding molecule. Such modifications include *inter alia* acetylation, acylation, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, cross-linking, disulfide bond formation, glycosylation, hydroxylation, methylation, oxidation, pegylation, proteolytic processing, phosphorylation, and the like. Alternatively, functional variants can be binding molecules as defined in the present invention comprising an amino acid sequence containing substitutions, insertions, deletions or combinations thereof of one or more amino acids compared to the amino acid sequences of the parental binding molecules. Furthermore, functional variants can comprise truncations of the amino acid sequence at either or both the amino or carboxyl termini. Functional variants according to the invention may have the same or different, either higher or lower, binding affinities compared to the parental binding molecule but are still capable of binding to the influenza virus or a fragment thereof. For instance, functional variants according to the invention may have increased or decreased binding affinities for an influenza virus or a fragment thereof compared to the parental binding molecules. Preferably, the amino acid sequences of the variable regions, including, but not limited to, framework regions, hypervariable regions, in particular the CDR3 regions, are modified. Generally, the light chain and the heavy chain variable regions comprise three hypervariable regions, comprising three CDRs, and more conserved regions, the so-called framework regions (FRs). The hypervariable regions comprise amino acid residues from CDRs and amino acid residues from hypervariable loops. Functional variants intended to fall within the scope of the present invention have at least about 50% to about 99%, preferably at least about 60% to about 99%, more preferably at least about 70% to about 99%, even more preferably at least about 80% to about 99%, most preferably at least about 90% to about 99%, in particular at least about 95% to about 99%, and in particular at least about 97% to about 99% amino acid sequence identity and/or homology with the parental binding molecules as defined herein. Computer algorithms such as *inter alia* Gap or Bestfit known to a person skilled in the art can be used to optimally align amino acid sequences to be compared and to define similar or identical amino acid residues. Functional variants can be obtained by altering the parental binding molecules or parts thereof by general molecular biology methods known in the art including, but not

limited to, error-prone PCR, oligonucleotide-directed mutagenesis, site-directed mutagenesis and heavy and/or light chain shuffling. In an embodiment the functional variants of the invention have neutralizing activity against influenza A viruses of group 1 and group 2, and/or influenza B viruses. The neutralizing activity may either be identical, or be higher or lower compared to the parental binding molecules. Henceforth, when the term (human) binding molecule is used, this also encompasses functional variants of the (human) binding molecule. Assays for verifying if a variant binding molecule has neutralizing activity are well known in the art (see WHO Manual on Animal Influenza Diagnosis and Surveillance, Geneva: World Health Organisation, 2005 version 2002.5).

In yet a further aspect, the invention includes immunoconjugates, *i.e.* molecules comprising at least one binding molecule as defined herein and further comprising at least one tag, such as *inter alia* a detectable moiety/agent. Also contemplated in the present invention are mixtures of immunoconjugates according to the invention or mixtures of at least one immunoconjugates according to the invention and another molecule, such as a therapeutic agent or another binding molecule or immunoconjugate. In a further embodiment, the immunoconjugates of the invention may comprise more than one tag. These tags can be the same or distinct from each other and can be joined/conjugated non-covalently to the binding molecules. The tag(s) can also be joined/conjugated directly to the human binding molecules through covalent bonding. Alternatively, the tag(s) can be joined/conjugated to the binding molecules by means of one or more linking compounds. Techniques for conjugating tags to binding molecules are well known to the skilled artisan.

The tags of the immunoconjugates of the present invention may be therapeutic agents, but they can also be detectable moieties/agents. Tags suitable in therapy and/or prevention may be toxins or functional parts thereof, antibiotics, enzymes, other binding molecules that enhance phagocytosis or immune stimulation. Immunoconjugates comprising a detectable agent can be used diagnostically to, for example, assess if a subject has been infected with an influenza virus or to monitor the development or progression of an influenza virus infection as part of a clinical testing procedure to, *e.g.*, determine the efficacy of a given treatment regimen. However, they may also be used for other detection and/or analytical and/or diagnostic purposes. Detectable moieties/agents

include, but are not limited to, enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals, and non-radioactive paramagnetic metal ions. The tags used to label the binding molecules for detection and/or analytical and/or diagnostic purposes depend on the specific detection/analysis/diagnosis techniques and/or methods used such as *inter alia* immunohistochemical staining of (tissue) samples, flow cytometric detection, scanning laser cytometric detection, fluorescent immunoassays, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), bioassays (*e.g.*, phagocytosis assays), Western blotting applications, etc. Suitable labels for the detection/analysis/diagnosis techniques and/or methods known in the art are well within the reach of the skilled artisan.

Furthermore, the human binding molecules or immunoconjugates of the invention can also be attached to solid supports, which are particularly useful for *in vitro* immunoassays or purification of influenza viruses or fragments thereof. Such solid supports might be porous or nonporous, planar or non-planar. The binding molecules of the present invention can be fused to marker sequences, such as a peptide to facilitate purification. Examples include, but are not limited to, the hexa-histidine tag, the hemagglutinin (HA) tag, the myc tag or the flag tag. Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate. In another aspect the binding molecules of the invention may be conjugated/attached to one or more antigens. Preferably, these antigens are antigens which are recognized by the immune system of a subject to which the binding molecule-antigen conjugate is administered. The antigens may be identical, but may also differ from each other. Conjugation methods for attaching the antigens and binding molecules are well known in the art and include, but are not limited to, the use of cross-linking agents. The binding molecules of the invention will bind to influenza virus HA and the antigens attached to the binding molecules will initiate a powerful T-cell attack on the conjugate, which will eventually lead to the destruction of the influenza virus.

Next to producing immunoconjugates chemically by conjugating, directly or indirectly, via for instance a linker, the immunoconjugates can be produced as fusion proteins comprising the binding molecules of the invention and a suitable tag. Fusion

proteins can be produced by methods known in the art such as, *e.g.*, recombinantly by constructing nucleic acid molecules comprising nucleotide sequences encoding the binding molecules in frame with nucleotide sequences encoding the suitable tag(s) and then expressing the nucleic acid molecules.

It is another aspect of the present invention to provide a nucleic acid molecule encoding at least a binding molecule, functional variant or immunoconjugate according to the invention. Such nucleic acid molecules can be used as intermediates for cloning purposes, *e.g.* in the process of affinity maturation as described above. In a preferred embodiment, the nucleic acid molecules are isolated or purified.

The skilled man will appreciate that functional variants of these nucleic acid molecules are also intended to be a part of the present invention. Functional variants are nucleic acid sequences that can be directly translated, using the standard genetic code, to provide an amino acid sequence identical to that translated from the parental nucleic acid molecules.

Preferably, the nucleic acid molecules encode binding molecules comprising the CDR regions as described above. In a further embodiment the nucleic acid molecules encode binding molecules comprising two, three, four, five or even all six CDR regions of the binding molecules of the invention.

In another embodiment, the nucleic acid molecules encode binding molecules comprising a heavy chain comprising the variable heavy chain sequences as described above. In another embodiment the nucleic acid molecules encode binding molecules comprising a light chain comprising the variable light chain sequences as described above. The nucleotide sequences and the amino acid sequences of the heavy and light chain variable regions of the binding molecules of the invention are given below.

It is another aspect of the invention to provide vectors, *i.e.* nucleic acid constructs, comprising one or more nucleic acid molecules according to the present invention. Vectors can be derived from plasmids such as *inter alia* F, R1, RP1, Col, pBR322, TOL, Ti, etc; cosmids; phages such as lambda, lambdoid, M13, Mu, P1, P22, Qβ, T-even, T-odd, T2, T4, T7, etc; plant viruses. Vectors can be used for cloning and/or for expression of the binding molecules of the invention and might even be used for gene therapy purposes. Vectors comprising one or more nucleic acid molecules according to the

invention operably linked to one or more expression-regulating nucleic acid molecules are also covered by the present invention. The choice of the vector is dependent on the recombinant procedures followed and the host used. Introduction of vectors in host cells can be effected by *inter alia* calcium phosphate transfection, virus infection, DEAE-dextran mediated transfection, lipofectamin transfection or electroporation. Vectors may be autonomously replicating or may replicate together with the chromosome into which they have been integrated. Preferably, the vectors contain one or more selection markers. The choice of the markers may depend on the host cells of choice, although this is not critical to the invention as is well known to persons skilled in the art. They include, but are not limited to, kanamycin, neomycin, puromycin, hygromycin, zeocin, thymidine kinase gene from Herpes simplex virus (HSV-TK), dihydrofolate reductase gene from mouse (dhfr). Vectors comprising one or more nucleic acid molecules encoding the human binding molecules as described above operably linked to one or more nucleic acid molecules encoding proteins or peptides that can be used to isolate the human binding molecules are also covered by the invention. These proteins or peptides include, but are not limited to, glutathione-S-transferase, maltose binding protein, metal-binding polyhistidine, green fluorescent protein, luciferase and beta-galactosidase.

Hosts containing one or more copies of the vectors mentioned above are an additional subject of the present invention. Preferably, the hosts are host cells. Host cells include, but are not limited to, cells of mammalian, plant, insect, fungal or bacterial origin. Bacterial cells include, but are not limited to, cells from Gram-positive bacteria or Gram-negative bacteria such as several species of the genera *Escherichia*, such as *E. coli*, and *Pseudomonas*. In the group of fungal cells preferably yeast cells are used. Expression in yeast can be achieved by using yeast strains such as *inter alia Pichia pastoris*, *Saccharomyces cerevisiae* and *Hansenula polymorpha*. Furthermore, insect cells such as cells from Drosophila and Sf9 can be used as host cells. Besides that, the host cells can be plant cells such as *inter alia* cells from crop plants such as forestry plants, or cells from plants providing food and raw materials such as cereal plants, or medicinal plants, or cells from ornamentals, or cells from flower bulb crops. Transformed (transgenic) plants or plant cells are produced by known methods, for example, Agrobacterium-mediated gene transfer, transformation of leaf discs, protoplast transformation by polyethylene glycol-

induced DNA transfer, electroporation, sonication, microinjection or bolistic gene transfer. Additionally, a suitable expression system can be a baculovirus system. Expression systems using mammalian cells, such as Chinese Hamster Ovary (CHO) cells, COS cells, BHK cells, NSO cells or Bowes melanoma cells are preferred in the present invention. Mammalian cells provide expressed proteins with posttranslational modifications that are most similar to natural molecules of mammalian origin. Since the present invention deals with molecules that may have to be administered to humans, a completely human expression system would be particularly preferred. Therefore, even more preferably, the host cells are human cells. Examples of human cells are *inter alia* HeLa, 911, AT1080, A549, 293 and HEK293T cells. In preferred embodiments, the human producer cells comprise at least a functional part of a nucleic acid sequence encoding an adenovirus E1 region in expressible format. In even more preferred embodiments, said host cells are derived from a human retina and immortalized with nucleic acids comprising adenoviral E1 sequences, such as 911 cells or the cell line deposited at the European Collection of Cell Cultures (ECACC), CAMR, Salisbury, Wiltshire SP4 OJG, Great Britain on 29 February 1996 under number 96022940 and marketed under the trademark PER.C6® (PER.C6 is a registered trademark of Crucell Holland B.V.). For the purposes of this application "PER.C6 cells" refers to cells deposited under number 96022940 or ancestors, passages up-stream or downstream as well as descendants from ancestors of deposited cells, as well as derivatives of any of the foregoing. Production of recombinant proteins in host cells can be performed according to methods well known in the art. The use of the cells marketed under the trademark PER.C6® as a production platform for proteins of interest has been described in WO 00/63403 the disclosure of which is incorporated herein by reference in its entirety.

In yet another embodiment, binding molecules of the present invention can also be produced in transgenic, non-human, mammals such as *inter alia* rabbits, goats or cows, and secreted into for instance the milk thereof.

In yet another alternative embodiment, binding molecules according to the present invention may be generated by transgenic non-human mammals, such as for instance transgenic mice or rabbits that express human immunoglobulin genes. Preferably, the transgenic non-human mammals have a genome comprising a human heavy chain

transgene and a human light chain transgene encoding all or a portion of the human binding molecules as described above. The transgenic non-human mammals can be immunized with a purified or enriched preparation of influenza virus or a fragment thereof. Protocols for immunizing non-human mammals are well established in the art. See Using Antibodies: A Laboratory Manual, Edited by: E. Harlow, D. Lane (1998), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York and Current Protocols in Immunology, Edited by: J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober (2001), John Wiley & Sons Inc., New York, the disclosures of which are incorporated herein by reference. Immunization protocols often include multiple immunizations, either with or without adjuvants such as Freund's complete adjuvant and Freund's incomplete adjuvant, but may also include naked DNA immunizations. In another embodiment, the human binding molecules are produced by B-cells, plasma and/or memory cells derived from the transgenic animals. In yet another embodiment, the human binding molecules are produced by hybridomas, which are prepared by fusion of B-cells obtained from the above-described transgenic non-human mammals to immortalized cells. B-cells, plasma cells and hybridomas as obtainable from the above-described transgenic non-human mammals and human binding molecules as obtainable from the above-described transgenic non-human mammals, B-cells, plasma and/or memory cells and hybridomas are also a part of the present invention.

In yet a further aspect, the invention provides compositions comprising at least a binding molecule, preferably a human monoclonal antibody, according to the invention, at least a functional variant thereof, at least an immunoconjugate according to the invention and/or a combination thereof. In addition to that, the compositions may comprise *inter alia* stabilizing molecules, such as albumin or polyethylene glycol, or salts. Preferably, the salts used are salts that retain the desired biological activity of the binding molecules and do not impart any undesired toxicological effects. If necessary, the human binding molecules of the invention may be coated in or on a material to protect them from the action of acids or other natural or non-natural conditions that may inactivate the binding molecules.

In yet a further aspect, the invention provides compositions comprising at least a nucleic acid molecule as defined in the present invention. The compositions may

comprise aqueous solutions such as aqueous solutions containing salts (*e.g.*, NaCl or salts as described above), detergents (*e.g.*, SDS) and/or other suitable components.

Furthermore, the present invention pertains to pharmaceutical compositions comprising at least a binding molecule such as a human monoclonal antibody of the invention (or functional fragment or variant thereof), at least an immunoconjugate according to the invention, at least a composition according to the invention, or combinations thereof. The pharmaceutical composition of the invention further comprises at least one pharmaceutically acceptable excipient. Pharmaceutically acceptable excipients are well known to the skilled person. The pharmaceutical composition according to the invention may further comprise at least one other therapeutic agent. Suitable agents are also well known to the skilled artisan.

In a preferred embodiment the pharmaceutical composition according to the invention comprises at least one additional binding molecule, *i.e.* the pharmaceutical composition can be a cocktail or mixture of binding molecules. The pharmaceutical composition may comprise at least two binding molecules according to the invention, or at least one binding molecule according to the invention and at least one further influenza virus binding and/or neutralizing molecule, such as another antibody directed against the HA protein or against other antigenic structures present on influenza viruses, such as M2. In another embodiment the additional binding molecule may be formulated for simultaneous separate or sequential administration.

In an embodiment the pharmaceutical compositions may comprise two or more binding molecules that have neutralizing activity against influenza A viruses and/or influenza B viruses. In an embodiment, the binding molecules exhibit synergistic neutralizing activity, when used in combination. As used herein, the term "synergistic" means that the combined effect of the binding molecules when used in combination is greater than their additive effects when used individually. The synergistically acting binding molecules may bind to different structures on the same or distinct fragments of influenza virus. A way of calculating synergy is by means of the combination index. The concept of the combination index (CI) has been described by Chou and Talalay (1984). The compositions may e.g. comprise one binding molecule having neutralizing activity

and one non-neutralizing binding molecule. The non-neutralizing and neutralizing binding molecules may also act synergistically in neutralizing influenza virus.

In an embodiment, the pharmaceutical composition may comprise at least one binding molecule according to the invention and at least one further influenza virus neutralizing binding molecule. The binding molecules in the pharmaceutical composition preferably are capable of reacting with influenza viruses of different subtypes. The binding molecules should be of high affinity and should have a broad specificity. Preferably, both binding molecules are cross-neutralizing molecules in that they each neutralize influenza viruses of different subtypes. In addition, preferably they neutralize as many strains of each of the different influenza virus subtypes as possible.

A pharmaceutical composition according to the invention can further comprise at least one other therapeutic, prophylactic and/or diagnostic agent. Preferably, the pharmaceutical composition comprises at least one other prophylactic and/or therapeutic agent. Preferably, said further therapeutic and/or prophylactic agents are agents capable of preventing and/or treating an influenza virus infection and/or a condition resulting from such an infection. Therapeutic and/or prophylactic agents include, but are not limited to, anti-viral agents. Such agents can be binding molecules, small molecules, organic or inorganic compounds, enzymes, polynucleotide sequences, anti-viral peptides, etc. Other agents that are currently used to treat patients infected with influenza viruses are M2 inhibitors (*e.g.*, amantidine, rimantadine) and/or neuraminidase inhibitors (*e.g.*, zanamivir, oseltamivir). These can be used in combination with the binding molecules of the invention. "In combination" herein means simultaneously, as separate formulations, or as one single combined formulation, or according to a sequential administration regimen as separate formulations, in any order. Agents capable of preventing and/or treating an infection with influenza virus and/or a condition resulting from such an infection that are in the experimental phase might also be used as other therapeutic and/or prophylactic agents useful in the present invention.

The binding molecules or pharmaceutical compositions of the invention can be tested in suitable animal model systems prior to use in humans. Such animal model systems include, but are not limited to, mouse, ferret and monkey.

Typically, pharmaceutical compositions must be sterile and stable under the conditions of manufacture and storage. The binding molecules, immunoconjugates, nucleic acid molecules or compositions of the present invention can be in powder form for reconstitution in the appropriate pharmaceutically acceptable excipient before or at the time of delivery. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Alternatively, the binding molecules, immunoconjugates, nucleic acid molecules or compositions of the present invention can be in solution and the appropriate pharmaceutically acceptable excipient can be added and/or mixed before or at the time of delivery to provide a unit dosage injectable form. Preferably, the pharmaceutically acceptable excipient used in the present invention is suitable to high drug concentration, can maintain proper fluidity and, if necessary, can delay absorption.

The choice of the optimal route of administration of the pharmaceutical compositions will be influenced by several factors including the physicochemical properties of the active molecules within the compositions, the urgency of the clinical situation and the relationship of the plasma concentrations of the active molecules to the desired therapeutic effect. For instance, if necessary, the binding molecules of the invention can be prepared with carriers that will protect them against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can *inter alia* be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Furthermore, it may be necessary to coat the binding molecules with, or co-administer the binding molecules with, a material or compound that prevents the inactivation of the human binding molecules. For example, the binding molecules may be administered to a subject in an appropriate carrier, for example, liposomes or a diluent.

The routes of administration can be divided into two main categories, oral and parenteral administration. The preferred administration route is intravenous or by inhalation.

Oral dosage forms can be formulated *inter alia* as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsions, hard capsules, soft gelatin capsules, syrups or elixirs, pills, dragees, liquids, gels, or slurries. These formulations can contain pharmaceutically excipients including, but not limited to, inert diluents, granulating and disintegrating agents, binding agents, lubricating agents, preservatives, colouring, flavouring or sweetening agents, vegetable or mineral oils, wetting agents, and thickening agents.

The pharmaceutical compositions of the present invention can also be formulated for parenteral administration. Formulations for parenteral administration can be *inter alia* in the form of aqueous or non-aqueous isotonic sterile non-toxic injection or infusion solutions or suspensions. The solutions or suspensions may comprise agents that are non-toxic to recipients at the dosages and concentrations employed such as 1,3-butanediol, Ringer's solution, Hank's solution, isotonic sodium chloride solution, oils, fatty acids, local anaesthetic agents, preservatives, buffers, viscosity or solubility increasing agents, water-soluble antioxidants, oil-soluble antioxidants and metal chelating agents.

In a further aspect, the binding molecules such as human monoclonal antibodies (functional fragments and variants thereof), immunoconjugates, compositions, or pharmaceutical compositions of the invention can be used as a medicament. So, a method of diagnosis, treatment and/or prevention of an influenza virus infection using the binding molecules, immunoconjugates, compositions, or pharmaceutical compositions of the invention is another part of the present invention. The above-mentioned molecules can *inter alia* be used in the diagnosis, prophylaxis, treatment, or combination thereof, of an influenza virus infection caused influenza viruses comprising HA of the H1, H2, H3, H4, H5, H6, H7, H8, H9, H10 and/or H11 subtype. In an embodiment, the above-mentioned molecules can also be used in the diagnosis, prophylaxis, treatment or combination thereof of an influenza virus infection caused by an influenza B virus. They are suitable for treatment of yet untreated patients suffering from an influenza virus infection and patients who have been or are treated for an influenza virus infection.

The above-mentioned molecules or compositions may be employed in conjunction with other molecules useful in diagnosis, prophylaxis and/or treatment. They can be used *in vitro, ex vivo* or *in vivo*. For instance, the binding molecules such as

human monoclonal antibodies (or functional variants thereof), immunoconjugates, compositions or pharmaceutical compositions of the invention can be co-administered with a vaccine against influenza virus (if available). Alternatively, the vaccine may also be administered before or after administration of the molecules of the invention. Instead of a vaccine, anti-viral agents can also be employed in conjunction with the binding molecules of the present invention. Suitable anti-viral agents are mentioned above.

The molecules are typically formulated in the compositions and pharmaceutical compositions of the invention in a therapeutically or diagnostically effective amount. Alternatively, they may be formulated and administered separately. For instance the other molecules such as the anti-viral agents may be applied systemically, while the binding molecules of the invention may be applied intravenously.

Treatment may be targeted at patient groups that are susceptible to influenza infection. Such patient groups include, but are not limited to e.g., the elderly (e.g. $\geq$ 50 years old, $\geq$ 60 years old, and preferably $\geq$ 65 years old), the young (e.g. $\leq$ 5 years old, $\leq$ 1 year old), hospitalized patients and already infected patients who have been treated with an antiviral compound but have shown an inadequate antiviral response.

Dosage regimens can be adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). A suitable dosage range may for instance be 0.01-100 mg/kg body weight, preferably 0.1-50 mg/kg body weight, preferably 0.01-15 mg/kg body weight. Furthermore, for example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. The molecules and compositions according to the present invention are preferably sterile. Methods to render these molecules and compositions sterile are well known in the art. The other molecules useful in diagnosis, prophylaxis and/or treatment can be administered in a similar dosage regimen as proposed for the binding molecules of the invention. If the other molecules are administered separately, they may be administered to a patient prior to (*e.g.*, 2 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 2 hrs, 4 hrs, 6 hrs, 8 hrs, 10 hrs, 12 hrs, 14 hrs, 16 hrs, 18 hrs, 20 hrs, 22 hrs, 24 hrs, 2 days, 3 days, 4 days, 5 days, 7 days, 2 weeks, 4 weeks or 6 weeks before), concomitantly with, or subsequent to (*e.g.*, 2 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 2 hrs, 4 hrs, 6 hrs, 8 hrs, 10 hrs, 12 hrs, 14 hrs, 16

hrs, 18 hrs, 20 hrs, 22 hrs, 24 hrs, 2 days, 3 days, 4 days, 5 days, 7 days, 2 weeks, 4 weeks or 6 weeks after) the administration of one or more of the human binding molecules or pharmaceutical compositions of the invention. The exact dosing regimen is usually sorted out during clinical trials in human patients.

Human binding molecules and pharmaceutical compositions comprising the human binding molecules are particularly useful, and often preferred, when to be administered to human beings as *in vivo* therapeutic agents, since recipient immune response to the administered antibody will often be substantially less than that occasioned by administration of a monoclonal murine, chimeric or humanized binding molecule.

In another aspect, the invention concerns the use of the binding molecules such as neutralizing human monoclonal antibodies (functional fragments and variants thereof), immunoconjugates, nucleic acid molecules, compositions or pharmaceutical compositions according to the invention in the preparation of a medicament for the diagnosis, prophylaxis, treatment, or combination thereof, of an influenza virus infection, in particular an influenza virus infection caused influenza viruses comprising HA of the H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, and/or H11 subtype and/or influenza B viruses.

Next to that, kits comprising at least a binding molecule such as a neutralizing human monoclonal antibody (functional fragments and variants thereof), at least an immunoconjugate, at least a nucleic acid molecule, at least a composition, at least a pharmaceutical composition, at least a vector, at least a host according to the invention or a combination thereof are also a part of the present invention. Optionally, the above-described components of the kits of the invention are packed in suitable containers and labelled for diagnosis, prophylaxis and/or treatment of the indicated conditions. The above-mentioned components may be stored in unit or multi-dose containers as an aqueous, preferably sterile, solution or as a lyophilised, preferably sterile, formulation for reconstitution. The containers may be formed from a variety of materials such as glass or plastic and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The kit may further comprise more containers comprising a pharmaceutically acceptable buffer. It may further include other materials desirable from a commercial and

user standpoint, including other buffers, diluents, filters, needles, syringes, culture medium for one or more of the suitable hosts and, possibly, even at least one other therapeutic, prophylactic or diagnostic agent. Associated with the kits can be instructions customarily included in commercial packages of therapeutic, prophylactic or diagnostic products, that contain information about for example the indications, usage, dosage, manufacture, administration, contra-indications and/or warnings concerning the use of such therapeutic, prophylactic or diagnostic products.

The binding molecules according to the present invention can also be advantageously used as a diagnostic agent in an *in vitro* method for the detection of influenza virus. The invention thus further pertains to a method of detecting influenza virus phylogenetic group 1 or group 2, or influenza B subtype influenza virus in a sample, wherein the method comprises the steps of (a) contacting a sample with a diagnostically effective amount of a binding molecule (functional fragments and variants thereof) or an immunoconjugate according to the invention, and (b) determining whether the binding molecule or immunoconjugate specifically binds to a molecule of the sample. The sample may be a biological sample including, but not limited to blood, serum, stool, sputum, nasophargyal aspirates, bronchial lavages, urine, tissue or other biological material from (potentially) infected subjects, or a non-biological sample such as water, drink, etc. The (potentially) infected subjects may be human subjects, but also animals that are suspected as carriers of influenza virus might be tested for the presence of the virus using the human binding molecules or immunoconjugates of the invention. The sample may first be manipulated to make it more suitable for the method of detection. Manipulation means *inter alia* treating the sample suspected to contain and/or containing the virus in such a way that the virus will disintegrate into antigenic components such as proteins, (poly)peptides or other antigenic fragments. Preferably, the human binding molecules or immunoconjugates of the invention are contacted with the sample under conditions which allow the formation of an immunological complex between the human binding molecules and the virus or antigenic components thereof that may be present in the sample. The formation of an immunological complex, if any, indicating the presence of the virus in the sample, is then detected and measured by suitable means. Such methods include, *inter alia*, homogeneous and heterogeneous binding immunoassays, such as radio-

immunoassays (RIA), ELISA, immunofluorescence, immunohistochemistry, FACS, BIACORE and Western blot analyses.

Preferred assay techniques, especially for large-scale clinical screening of patient sera and blood and blood-derived products are ELISA and Western blot techniques. ELISA tests are particularly preferred. For use as reagents in these assays, the binding molecules or immunoconjugates of the invention are conveniently bonded to the inside surface of microtiter wells. The binding molecules or immunoconjugates of the invention may be directly bonded to the microtiter well. However, maximum binding of the binding molecules or immunoconjugates of the invention to the wells might be accomplished by pre-treating the wells with polylysine prior to the addition of the binding molecules or immunoconjugates of the invention. Furthermore, the binding molecules or immunoconjugates of the invention may be covalently attached by known means to the wells. Generally, the binding molecules or immunoconjugates are used in a concentration between 0.01 to 100 µg/ml for coating, although higher as well as lower amounts may also be used. Samples are then added to the wells coated with the binding molecules or immunoconjugates of the invention.

Furthermore, binding molecules of the invention can be used to identify specific binding structures of influenza virus. The binding structures can be epitopes on proteins and/or polypeptides. They can be linear, but also structural and/or conformational. In one embodiment, the binding structures can be analysed by means of PEPSCAN analysis (see *inter alia* WO 84/03564, WO 93/09872, Slootstra *et al.*, 1996). Alternatively, a random peptide library comprising peptides from a protein of influenza virus can be screened for peptides capable of binding to the binding molecules of the invention.

The invention is further illustrated in the following examples and figures. The examples are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1 *Construction of scFv phage display libraries using RNA extracted from peripheral blood mononuclear cells*

Peripheral blood was collected from normal healthy donors by venapuncture in EDTA anti-coagulation sample tubes. scFv phage display libraries were obtained as described in WO 2008/028946, which is incorporated by reference herein. RNA was isolated from peripheral blood mononuclear cells and cDNA prepared. A two round PCR amplification approach was applied using the primer sets shown in Tables 1 and 2 to isolate the immunoglobulin VH and VL regions from the respective donor repertoire.

First round amplification on the respective cDNA using the primer sets mentioned in Table 1 yielded 7, 6 and 9 products of about 650 base pairs for respectively VH, Vkappa and Vlambda regions. For IgM VH region amplification the OCM constant primer was used in combination with OH1 to OH7. The thermal cycling program for first round amplifications was: 2 min 96°C (denaturation step), 30 cycles of 30 sec 96°C/ 30 sec 60°C/ 60 sec 72°C, 10 min 72°C final elongation and 6°C refrigeration. The products were loaded on and isolated from a 1% agarose gel using gel-extraction columns (Macherey Nagel) and eluted in 50 µl 5 mM Tris-HCl pH 8.0. Ten percent of first round products (3 to 5 µl) was subjected to second round amplification using the primers mentioned in Table 2. These primers were extended with restriction sites enabling the directional cloning of the respective VL and VH regions into phage display vector PDV-C06. The PCR program for second round amplifications was as follows: 2 min 96°C (denaturation step), 30 cycles of 30 sec 96°C/ 30 sec 60°C/ 60 sec 72°C, 10 min 72°C final elongation and 6°C refrigeration. The second round products (~350 base pairs) were first pooled according to natural occurrence of J segments found in immunoglobulin gene products, resulting in 7, 6 and 9 pools for respectively the VH, Vkappa and Vlambda variable regions (see Tables 3 and 4). To obtain a normalized distribution of immunoglobulin sequences in the immune library the 6 Vkappa and 9 Vlambda light chain pools were mixed according to the percentages mentioned in Table 3. This single final VL pool (3 µg) was digested overnight with SalI and NotI restriction enzymes, loaded on and isolated from a 1% agarose gel (~350 base pairs) using Macherey Nagel gel-extraction columns and ligated in SalI-NotI cut PDV-C06 vector (~5000 base pairs) as follows: 10 µl PDV-C06 vector (50 ng/µl), 7 µl VL insert (10 ng/µl), 5 µl 10X ligation buffer (NEB), 2.5 T4 DNA Ligase (400 U/µl) (NEB), 25.5 µl ultrapure water (vector to insert ratio was 1:2). Ligation was performed overnight in a water bath of 16°C. Next, the

volume was doubled with water, extracted with an equal volume of phenol-chloroform-isoamylalcohol (75:24:1) (Invitrogen) followed by chloroform (Merck) extraction and precipitated with 1 µl Pellet Paint (Novogen), 10 µl sodium acetate (3 M pH 5.0) and 100 µl isopropanol for 2 hrs at -20°C. The obtained sample was subsequently centrifuged at 20.000xg for 30 min at 4°C. The obtained precipitate was washed with 70% ethanol and centrifuged for 10 min at 20.000xg at room temperature. Ethanol was removed by vacuum aspiration and the pellet was air dried for several min and then dissolved in 50 µl buffer containing 10 mM Tris-HCl, pH 8.0. 2 µl ligation mixture was used for the transformation of 40 µl TG-1 electro-competent cells (Agilent) in a chilled 0.1 cm electroporation cuvette (Biorad) using a Genepulser II apparatus (Biorad) set at 1.7 kV, 200 Ohm, 25 µF (time constant ~4,5 msec). Directly after pulse, the bacteria were flushed from the cuvette with 1000 µl SOC medium (Invitrogen) containing 5% (w/v) glucose (Sigma) at 37°C and transferred to a 15 ml round bottom culture tube. Another 500 µl SOC/glucose was used to flush residual bacteria from the cuvette and was added to the culture tube. Bacteria were recovered by culturing for exactly one hr at 37°C in a shaker incubator at 220 rpm. The transformed bacteria were plated over large 240 mm square petridishes (NUNC) containing 150 ml 2TY agar (16 g/l bacto-tryptone, 10 g/l bacto-yeast extract, 5 g/l NaCl, 15 g/l agar, pH 7.0) supplemented with 50 µg/ml ampicillin and 5% (w/v) glucose (Sigma). A 1 to 1000 dilution was plated for counting purposes on 15 cm petridishes containing the same medium. This transformation procedure was repeated sequentially ten times and the complete each transformation was plated on a separate square petridish and grown overnight in a 37°C culture stove. Typically, around $1x10^7$ cfu ($1x10^6$ per petridish) were obtained using the above protocol. The intermediate VL light chain library was harvested from the plates by mildly scraping the bacteria into 10 ml 2TY medium per plate. The cell mass was determined by OD600 measurement and two times 500 OD of bacteria was used for maxi plasmid DNA preparation using two P500 maxiprep columns (Macherey Nagel) according to manufacturer's instructions.

Analogous to the VL variable regions, the second round VH-JH products were first mixed together to obtain the normal J segment usage distribution (see Table 4), resulting in 7 VH subpools called PH1 to PH7. The pools were mixed to acquire a normalized sequence distribution using the percentages depicted in Table 4, obtaining

one VH fraction that was digested with SfiI and XhoI restriction enzymes and ligated in SfiI-XhoI cut PDV-VL intermediate library obtained as described above. The ligation set-up, purification method, subsequent transformation of TG1 and harvest of bacteria was essentially as described for the VL intermediate library (see above) with the exception that 20 transformations and 20 square petridishes were used. The final library (approximately $1x10^7$ cfu) was checked for insert frequency with a colony PCR using a primer set flanking the inserted VH-VL regions. 90% of the colonies showed a correct length insert. The colony PCR products were used for subsequent DNA sequence analysis to check sequence variation and to assess the percentage of colonies showing a complete ORF. This was 76% . Finally, the library was rescued and amplified by using CT helper phages (see WO 02/103012) and was used for phage antibody selection by panning methods as described below.

Example 2

Selection of phages carrying single chain Fv fragments against Influenza A and Influenza B heamagglutinin

Antibody fragments were selected using antibody phage display libraries constructed essentially as described above and general phage display technology and MABSTRACT® technology essentially as described in US Patent Number 6,265,150 and in WO 98/15833 (both of which are incorporated by reference herein). Furthermore, the methods and helper phages as described in WO 02/103012 (which is incorporated by reference herein) were used in the present invention.

Selection was performed against recombinant hemagglutinin (HA) of influenza A subtype H1 (A/New Caledonia/20/99), H3 (A/Wisconsin/67/2005), H4 (A/Duck/Hong Kong/24/1976), H5 (A/Chicken/Vietnam/28/2003), H7 (A/Netherlands/219/2003) and H9 (A/HongKong/1073/99). HA antigens were diluted in PBS (5.0 µg/ml), added to MaxiSorp™ Nunc-Immuno Tubes (Nunc) and incubated overnight at 4°C on a rotating wheel. The immunotubes were emptied and washed three times in block buffer (2% non-fat dry milk (ELK) in PBS). Subsequently, the immunotubes were filled completely with block buffer and incubated for 1-2 hrs at room temperature. Aliquots of phage display library (500-1000 µl, $0.5x10^{13}$ – $1x10^{13}$ cfu, amplified using CT helper phage (see WO

02/103012)) were blocked in blocking buffer supplemented with 10% non-heat inactivated fetal bovine serum and 2% mouse serum for 1-2 hrs at room temperature. The blocked phage library was added to the immunotubes, incubated for 2 hrs at room temperature, and washed with wash buffer (0.05% (v/v) Tween-20 in PBS) to remove unbound phages. Bound phages were eluted from the respective antigen by incubation with 1 ml of 100 mM triethylamine (TEA) for 10 min at room temperature. Subsequently, the eluted phages were mixed with 0.5 ml of 1 M Tris-HCl pH 7.5 to neutralize the pH. This mixture was used to infect 5 ml of an XL1-Blue *E.coli* culture that had been grown at 37°C to an OD 600 nm of approximately 0.3. The phages were allowed to infect the XL1-Blue bacteria for 30 min at 37°C. Then, the mixture was centrifuged for 10 min at 3000xg at room temperature and the bacterial pellet was resuspended in 0.5 ml 2-trypton yeast extract (2TY) medium. The obtained bacterial suspension was divided over two 2TY agar plates supplemented with tetracycline, ampicillin and glucose. After incubation overnight of the plates at 37°C, the colonies were scraped from the plates and used to prepare an enriched phage library, essentially as described by De Kruif *et al.* (1995) and WO 02/103012. Briefly, scraped bacteria were used to inoculate 2TY medium containing ampicillin, tetracycline and glucose and grown at a temperature of 37°C to an OD 600 nm of ~0.3. CT helper phages were added and allowed to infect the bacteria after which the medium was changed to 2TY containing ampicillin, tetracycline and kanamycin. Incubation was continued overnight at 30°C. The next day, the bacteria were removed from the 2TY medium by centrifugation after which the phages in the medium were precipitated using polyethylene glycol (PEG) 6000/NaCl. Finally, the phages were dissolved in 2 ml of PBS with 1% bovine serum albumin (BSA), filter-sterilized and used for the next round of selection. The second round of selection is performed either on the same HA subtype and/or on HA of a different subtype.

Two consecutive rounds of selections were performed before isolation of individual single-chain phage antibodies. After the second round of selection, individual *E.coli* colonies were used to prepare monoclonal phage antibodies. Essentially, individual colonies were grown to log-phase in 96 well plate format and infected with VCS-M13 helper phages after which phage antibody production was allowed to proceed overnight. Phagemids were sequence analysed and all unique phagemids were used for further

analysis. The supernatants containing phage antibodies were used directly in ELISA for binding to HA antigens. Alternatively, phage antibodies were PEG/NaCl-precipitated and filter-sterilized for both elisa and flow cytometry analysis.

Example 3

Validation of the HA specific single-chain phage antibodies

Selected supernatants containing single-chain phage antibodies that were obtained in the screenings described above were validated in ELISA for specificity, *i.e.* binding to different HA antigens. For this purpose, baculovirus expressed recombinant H1 (A/New Caledonia/20/99), H3 (A/Wisconsin/67/2005), H5 (A/Vietnam/1203/04) H7 (A/Netherlands/219/2003), and B (B/Ohio/01/2005) HAs (Protein Sciences, CT, USA) were coated to Maxisorp™ ELISA plates. After coating, the plates were washed three times with PBS containing 0.1% v/v Tween-20 and blocked in PBS containing 3% BSA or 2% ELK for 1 hr at room temperature. The selected single-chain phage antibodies were incubated for 1 hr in an equal volume of PBS containing 4% ELK to obtain blocked phage antibodies. The plates were emptied, washed three times with PBS/0.1% Tween-20 and the blocked single-chain phage antibodies were added to the wells. Incubation was allowed to proceed for one hr, the plates were washed with PBS/0.1% Tween-20 and bound phage antibodies were detected (using OD 492nm measurement) using an anti-M13 antibody conjugated to peroxidase. As a control, the procedure was performed simultaneously without single-chain phage antibody and with an unrelated negative control single-chain phage antibody. From the selections on the different HA antigens with the phage libraries, 13 unique single-chain phage antibodies specifically binding recombinant influenza A H1, H3, H5, H7 and influenza B HA were obtained (SC09-003, SC09-004, SC09-005, SC09-006, SC09-007, SC09-008, SC09-009, SC09-010, SC09-011, SC09-030, SC09-112, SC09-113 and SC09-114). See Table 5.

Alternatively, PEG/NaCl-precipitated and filter-sterilized phage antibodies were used to validate binding and specificity by FACS analysis. For this purpose, full-length recombinant influenza A subtypes H1 (A/New Caledonia/20/1999), H3 (A/Wisonsin/67/2005) and H7 (A/Netherlands/219/2003) HAs were expressed on the surface of PER.C6 cells. The cells were incubated with single-chain phage antibodies for

1 hr followed by three wash steps with PBS+0.1%BSA. Bound phages were detected using FITC conjugated M13-antibody. From the selections on the different HA antigens with the phage libraries, 14 single-chain phage antibodies specifically binding influenza A subtypes H1, H3 and H7 HA were found (SC09-003, SC09-004, SC09-005, SC09-006, SC09-007, SC09-008, SC09-009. SC09-010, SC09-011, SC09-012, SC09-030, SC09-112, SC09-113 and SC09-114). See table 6.

All 16 phage antibodies, SC09-003, SC09-004, SC09-005, SC09-006, SC09-007, SC09-008, SC09-009. SC09-010, SC09-011, SC09-012, SC09-029, SC09-030, SC09-031, SC09-112, SC09-113 and SC09-114, were used for construction of fully human immunoglobulins.

Example 4

Construction of fully human immunoglobulin molecules (human monoclonal antibodies) from the selected single chain Fvs

From the selected specific single-chain phage antibodies (scFv) clones plasmid DNA was obtained and nucleotide and amino acid sequences were determined according to standard techniques. Heavy and light chain variable regions of the scFvs were cloned directly by restriction digest for expression in the IgG expression vectors pIg-C911-HCgamma1 (see SEQ ID N0: 175), pIG-C909-Ckappa (see SEQ ID NO: 176), or pIg-C910-Clambda (see SEQ ID No: 177). The VH and VL gene identity (see Tomlinson IM et al. V-BASE Sequence Directory. Cambridge United Kingdom: MRC Centre for Protein Engineering (1997)) of the scFvs were determined (see Table 7).

Nucleotide sequences for all constructs were verified according to standard techniques known to the skilled artisan. The resulting expression constructs encoding the human IgG1 heavy and light chains were transiently expressed in combination in 293T cells and supernatants containing human IgG1 antibodies were obtained and produced using standard purification procedures.

The amino acid sequence of the CDRs of the heavy and light chains of the selected immunoglobulin molecules is given in Table 7.

The number of amino-acid differences and the % identity of all heavy and light chain variable domains is given in Table 8.

Example 5

Cross-binding reactivity of IgGs

A panel of five of the IgG antibodies described above, CR9005, CR9030, CR9112, CR9113 and CR9114, was validated in ELISA for binding specificity, *i.e.* binding to different HA antigens. For this purpose, baculovirus expressed recombinant H1 (A/New Caledonia/20/1999), H3 (A/Wisconsin/67/2005), H5 (A/Vietnam/1203/04, H7 (A/Netherlands/219/2003) and H9 (A/HongKong/1073/99) HA's (Protein Sciences, CT, USA) were coated to Maxisorp™ ELISA plates. After coating, the plates were washed three times with PBS containing 0.1% v/v Tween-20 and blocked in PBS containing 3% BSA or 2% ELK for 1 hr at room temperature. The plates were emptied, washed three times with PBS/0.1% Tween-20 and the IgG antibodies were added to the wells. Incubation was allowed to proceed for one hr, the plates were washed with PBS/0.1% Tween-20 and bound antibodies were detected (using OD 492nm measurement) using an anti-human IgG antibody conjugated to peroxidase. As a control, an unrelated IgG CR4098 was used.

CR9005, CR9030, CR9112, CR9113 and CR9114 were shown to have heterosubtypic cross-binding activity to all the recombinant HAs tested. See table 9.

Additionally, the selected antibodies were used to test heterosubtypic binding by FACS analysis. For this purpose, full-length recombinant influenza A subtypes H1 (A/New Caledonia/20/1999), H3 (A/Wisonsin/67/2005) and H7 (A/Netherlands/219/2003) HAs were expressed on the surface of PER.C6 cells. The cells were incubated with IgG antibodies for 1 hr followed by three wash steps with PBS+0.1%BSA. Bound antibodies were detected using PE conjugated anti-human antibody. As a control, untransfected PER.C6 cells were used. CR9005, CR9030, CR9112, CR9113 and CR9114 show cross-binding activity to influenza A subtypes H1, H3 and H7 HA but not wild-type PER.C6 cells. See table 9.

Example 6

Cross-neutralizing activity of IgGs

In order to determine whether the selected IgGs were capable of blocking multiple influenza A strains, additional *in vitro* virus neutralization assays (VNA) were performed. The VNA were performed on MDCK cells (ATCC CCL-34). MDCK cells were cultured in MDCK cell culture medium (MEM medium supplemented with antibiotics, 20 mM Hepes and 0.15% (w/v) sodium bicarbonate (complete MEM medium), supplemented with 10% (v/v) fetal bovine serum). The H1 (A/WSN/33, A/New Caledonia/20/1999, A/Solomon Islands/IVR-145 (high-growth reassortant of A/Solomon Islands/3/2006), A/Brisbane/59/2007, A/NYMC/X-181 (high-growth reassortant of A/California/07/2009), H2 (A/Env/MPU3156/05), H3 (A/Hong Kong/1/68, A/Johannesburg/33/94, A/Panama/2000/1999, A/Hiroshima/52/2005, A/Wisconsin/67/2005 and A/Brisbane/10/2007), H4 (A/WF/HK/MPA892/06), H5 (PR8-H5N1-HK97 (6:2 reassortant of A/Hong Kong/156/97 and A/PR/8/34) and A/Eurasian Wigeon/MPF461/07), H6 (A/Eurasian Wigeon/MPD411/07), H7 (NIBRG-60 (6:2 reassortant of A/Mallard/Netherlands/12/2000) and PR8-H7N7-NY (7:1 reassortant of A/New York/107/2003 (H7N7) and A/PR/8/34)), H8 (A/Eurasian Wigeon/MPH571/08) H9 (A/Hong Kong/1073/99 and A/Chick/HK/SSP176/09), H10 (A/Chick/Germany/N/49) and H14 (PR8-H14N5 (6:2 reassortant of A/mallard/Astrakhan/263/1982 (H14N5) and A/PR/8/34)) strains which were used in the assay were all diluted to a titer of 5,7 $x10^3$ TCID50/ml (50% tissue culture infective dose per ml), with the titer calculated according to the method of Spearman and Karber. The IgG preparations (200 µg/ml) were serially 2-fold diluted (1:2 - 1:512) in complete MEM medium in quadruplicate wells. 25 µl of the respective IgG dilution was mixed with 25 µl of virus suspension (100 TCID50/25 µl) and incubated for one hr at 37°C. The suspension was then transferred in quadruplicate onto 96-well plates containing confluent MDCK cultures in 50 µl complete MEM medium. Prior to use, MDCK cells were seeded at $3x10^4$ cells per well in MDCK cell culture medium, grown until cells had reached confluence, washed with 300-350 µl PBS, pH 7.4 and finally 50 µl complete MEM medium was added to each well. The inoculated cells were cultured for 3-4 days at 37°C and observed daily for the development of cytopathogenic effect (CPE). CPE was compared to the positive control.

CR9005, CR9112, CR9113 and CR9114 show heterosubtypic cross-neutralizing activity to representative strains of all tested influenza A subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9 and H10 viruses. See table 10.

Example 7

Pan-influenza antibodies bind to the pre-fusion conformation of HA

In order to determine whether the selected IgGs were capable of binding the pre- or post-fusion conformation of the HA molecule, an in vitro pH-shift experiment was performed. For this purpose, full-length recombinant influenza A subtypes H1 (A/New Caledonia/20/99), H3 (A/Wisonsin/67/2005), H5 (A/Vietnam/1203/04), H7 (A/Netherlands/219/03) and H9 (A/Hong Kong/1073/99) HA were expressed on the surface of PER.C6 cells. To measure mAb binding to different structural HA conformations, cells were detached from the plastic support using PBS-EDTA and subsequently treated with trypsin (TrypLE^{TM}Select, Gibco) for 5 min at RT, washed (1% BSA in PBS) and incubated for 15 min in citric acid–sodium phosphate buffer (pH 4.9). Cell samples were set aside after each processing step (untrypsinized/HA0; trypsinized/HA1-HA2; pH 4.9/fusion HA) and fractions of each treatment were incubated with mAb CR9114 for 1 hour. Cells were then incubated for 30 min with phycoerythrin-conjugated anti-human IgG (Southern Biotech) in 1% BSA. Stained cells were analysed using a FACS Canto with FACS Diva software (Becton Dickinson).
FACS binding of IgG1s to surface expressed HA was after sequential treatment with trypsin and pH 4.9 buffered medium and expressed as percentage binding to untreated HA (A). See Figure 1A.

Antibody CR9114 shows a marked decrease in binding after pH-shift indicating specificity for an epitope present only before the low pH induced conformational change of the HA molecule.

Alternatively, to test whether the IgGs can block the low pH induced conformational change of HA, antibody CR9114 was added before the low pH step. Samples of consecutive treatments were split and stained with either phycoerythrin-conjugated anti-human IgG (Southern Biotech). Stained cells were analysed using a FACS Canto with FACS Diva software (Becton Dickinson). See Fig 1B.

Antibody CR9114 shows a high level of residual binding to the various HAs after pH-shift indicating that when these antibodies are bound to the HA molecule, the low pH induced conformational change does not occur.

Example 8

Affinity measurements of Fabs on various influenza A and B HAs.

Recombinant soluble HA of A/New Caledonia/20/1999 (H1), A/Brisbane/59/2007 (H1), A/Wisconsin/67/2005 (H3), A/Brisbane/10/2007 (H3, B/Florida/4/2006 (B), B/Brisbane/60/2008 (B) and B/Malaysia/2506/2004 (B) produced using baculovirus vectors in insect cells were purchased from Protein Sciences Corp (CT, USA) and biotinylated at room temperature (RT) for 40 min using EZ-link sulfo-NHS-LC-LC-biotin (Pierce). Buffer exchange step to PBS was performed using Amicon Ultra 0.5 ml Centrifugal Filters (Millipore). Biotinylated HA was bound to Streptavidin sensors at 37 °C for 1200 seconds. Association of Fab fragment of CR9005, CR9112, CR9113 and CR9114 to HA was measured on Octet QK (ForteBio) for 700 seconds at 37 °C by exposing the sensors to 100 nM antibody in 1x kinetic buffer (ForteBio). Dissociation of the Fab fragments was assessed by exposing the sensors to 1x kinetic buffer for 9000 seconds at 37 °C. Fab fragments of CR9005, CR9112, CR9113 and CR9114 all bind with micro- to pico-molar affinities to H1, H3 and influenza B HA.

Example 9

Competition for binding with other stem binding antibodies

Recombinant soluble HA of A/New Caledonia/20/1999 (H1N1) and A/Wisconsin/67/2005 (H3N2) produced using baculovirus vectors in insect cells were purchased from Protein Sciences Corp (CT, USA) and biotinylated at room temperature (RT) for 40 min using EZ-link sulfo-NHS-LC-LC-biotin (Pierce). Buffer exchange step to PBS was performed using Amicon Ultra 0.5 ml Centrifugal Filters (Millipore). Biotinylated HA was bound to Streptavidin sensors at 37 °C for 1200 seconds. Association of antibodies CR9114 and CR6261 to H1 HA was measured on Octet QK (ForteBio) for 700 seconds at 37 °C by exposing the sensors to 100 nM antibody in 1x

kinetic buffer (ForteBio) after which the degree of additional binding was assessed by exposing the sensors to a second antibody (100 nM in 1x kinetic buffer) in the presence of the first antibody (100 nM) for 700 seconds at 37 °C. As a control, mAb CR9020, binding to the globular head of H1 was taken along. Association of antibodies CR9114 and CR8020 to H3 HA was measured on Octet QK (ForteBio) for 900 seconds at 37 °C by exposing the sensors to 100 nM antibody in 1x kinetic buffer (ForteBio) after which the degree of additional binding was assessed by exposing the sensors to a second antibody (100 nM in 1x kinetic buffer) in the presence of the first antibody (100 nM) for 900 seconds at 37 °C. As a control, mAb CR8057, binding to the globular head of H3 was taken along.

CR9114 competes for binding to H1 HA with CR6261 and to H3 HA with CR8020. CR9114 therefore likely binds an epitope overlapping with both the epitopes of CR6261 and CR8020 in the stem-region of HA. (See Fig. 2)

Example 10

Prophylactic activity of human IgG monoclonal antibody CR9114 against lethal influenza B challenge in vivo

A study was performed to test the prophylactic effect of the monoclonal antibody CR9114 against a lethal challenge with influenza B virus in vivo. MAb CR9114 was tested for prophylactic efficacy in a mouse lethal challenge model with mouse adapted influenza B/Florida/04/2006 virus (Central Veterinary Institute (CVI), Lelystad, The Netherlands). The B/Florida/04/2006 virus was adapted to mice after 5 lung-to-lung passages. The mouse adapted influenza B passage 5 virus was propagated in embryonated chicken eggs in CVI's laboratory. All mice (Balb/c, female, age 6-8 weeks, n=10 per group) were acclimatized and maintained for a period of at least 4 days prior to the start of the experiment. MAb CR9114 was dosed at 15 mg/kg intravenously in the tail vein (*vena coccygeus*) at day -1 before challenge, assuming an average weight of 18 g per mouse and a fixed dose volume of 0.2 mL. A control group was taken along dosed with vehicle control. The mice were then challenged at day 0 with 25 LD_{50} B/Florida/04/2006 influenza B virus by intranasal inoculation. Clinical signs and body weights were determined daily from day-1 before challenge until day 8. Clinical signs were scored with

a scoring system (0=no clinical signs; 1=rough coat; 2=rough coat, less reactive during handling; 3=rough coat, rolled up, laboured breathing, less reactive during handling; 4=rough coat, rolled up, laboured breathing, inactive response to manipulation/handlings). At a score of 4 the animal was euthanized.

All mice were active and appeared healthy without showing signs of disease during the acclimatization period. Fig. 3A shows the survival rates of the mice, following mAb administration. Mice dosed with 15 mg/kg mAb CR9114 showed a survival rate of 100%, whereas in the control mAb group 50% survived.

In Figure 3B the mean body weight change of the mice during the 8 day study period following mAb administration is shown. In the mAb CR9114 group the mice did not loose weight over the 8 day study period, whereas in the vehicle control group weight loss was observed. Median clinical scores of the mice are depicted in Fig. 3C. Of the mice treated with 15 mg/kg mAb CR9114 at day -1 pre-challenge, all survived and none of the animals showed any clinical signs during the observation period (from day 0 to day 8 post infection). These results show that the human anti influenza antibody CR9114, identified and developed as disclosed herein, is able to provide protection against a lethal dose of influenza B virus *in vivo*. When administered one day prior to infection at a dose of 15 mg/kg or higher, mAb CR9114 was able to completely prevent clinical manifestation of influenza B infection in mice.

Table 1. First round Vkappa, Vlambda and VH amplifications

Primer name	Primer nucleotide sequence	SEQ ID NO:
OK1 (HuVK1B)	GAC ATC CAG WTG ACC CAG TCT CC	65
OK2 (HuVK2)	GAT GTT GTG ATG ACT CAG TCT CC	66
OK3 (HuVK2B2)	GAT ATT GTG ATG ACC CAG ACT CC	67
OK4 (HuVK3B)	GAA ATT GTG WTG ACR CAG TCT CC	68
OK5 (HuVK5)	GAA ACG ACA CTC ACG CAG TCT CC	69
OK6 (HuVK6)	GAA ATT GTG CTG ACT CAG TCT CC	70
OCK (HuCK)	ACA CTC TCC CCT GTT GAA GCT CTT	71
OL1 (HuVL1A)*	CAG TCT GTG CTG ACT CAG CCA CC	72
OL1 (HuVL1B)*	CAG TCT GTG YTG ACG CAG CCG CC	73
OL1 (HuVL1C)*	CAG TCT GTC GTG ACG CAG CCG CC	74
OL2 (HuVL2B)	CAG TCT GCC CTG ACT CAG CC	75
OL3 (HuVL3A)	TCC TAT GWG CTG ACT CAG CCA CC	76
OL4 (HuVL3B)	TCT TCT GAG CTG ACT CAG GAC CC	77
OL5 (HuVL4B)	CAG CYT GTG CTG ACT CAA TC	78
OL6 (HuVL5)	CAG GCT GTG CTG ACT CAG CCG TC	79
OL7 (HuVL6)	AAT TTT ATG CTG ACT CAG CCC CA	80
OL8 (HuVL7/8)	CAG RCT GTG GTG ACY CAG GAG CC	81
OL9 (HuVL9)#	CWG CCT GTG CTG ACT CAG CCM CC	82
OL9 (HuVL10)#	CAG GCA GGG CTG ACT CAG	83
OCL (HuCL2)X	TGA ACA TTC TGT AGG GGC CAC TG	84
OCL (HuCL7)X	AGA GCA TTC TGC AGG GGC CAC TG	85
OH1(HuVH1B7A)+	CAG RTG CAG CTG GTG CAR TCT GG	86
OH1 (HuVH1C)+	SAG GTC CAG CTG GTR CAG TCT GG	87
OH2 (HuVH2B)	CAG RTC ACC TTG AAG GAG TCT GG	88
OH3 (HuVH3A)	GAG GTG CAG CTG GTG GAG	89
OH4 (HuVH3C)	GAG GTG CAG CTG GTG GAG WCY GG	90
OH5 (HuVH4B)	CAG GTG CAG CTA CAG CAG TGG GG	91
OH6 (HuVH4C)	CAG STG CAG CTG CAG GAG TCS GG	92
OH7 (HuVH6A)	CAG GTA CAG CTG CAG CAG TCA GG	93
OCM (HuCIgM)	TGG AAG AGG CAC GTT CTT TTC TTT	94

* Mix in 1:1:1 ratio
\# Mix in 1:1 ratio
X Mix in 1:1 ratio
+ Mix in 1:1 ratio

Table 2. Second round Vkappa, Vlambda and VH amplifications

Primer name	Primer nucleotide sequence	SEQ ID NO
OK1S (HuVK1B-SAL)	TGA GCA CAC AGG TCG ACG GAC ATC CAG WTG ACC CAG TCT CC	95
OK2S (HuVK2-SAL)	TGA GCA CAC AGG TCG ACG GAT GTT GTG ATG ACT CAG TCT CC	96
OK3S (HuVK2B2-SAL)	TGA GCA CAC AGG TCG ACG GAT ATT GTG ATG ACC CAG ACT CC	97
OK4S (HuVK3B-SAL)	TGA GCA CAC AGG TCG ACG GAA ATT GTG WTG ACR CAG TCT CC	98
OK5S (HuVK5-SAL)	TGA GCA CAC AGG TCG ACG GAA ACG ACA CTC ACG CAG TCT CC	99
OK6S (HuVK6-SAL)	TGA GCA CAC AGG TCG ACG GAA ATT GTG CTG ACT CAG TCT CC	100
OJK1 (HuJK1-NOT)	GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT TTC CAC CTT GGT CCC	101
OJK2 (HuJK2-NOT)	GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAG CTT GGT CCC	102
OJK3 (HuJK3-NOT)	GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT ATC CAC TTT GGT CCC	103
OJK4 (HuJK4-NOT)	GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAC CTT GGT CCC	104
OJK5 (HuJK5-NOT)	GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT AAT CTC CAG TCG TGT CCC	105
OL1S (HuVL1A-SAL)*	TGA GCA CAC AGG TCG ACG CAG TCT GTG CTG ACT CAG CCA CC	106
OL1S (HuVL1B-SAL)*	TGA GCA CAC AGG TCG ACG CAG TCT GTG YTG ACG CAG CCG CC	107
OL1S (HuVL1C-SAL)*	TGA GCA CAC AGG TCG ACG CAG TCT GTC GTG ACG CAG CCG CC	108
OL2S (HuVL2B-SAL)	TGA GCA CAC AGG TCG ACG CAG TCT GCC CTG ACT CAG CC	109
OL3S (HuVL3A-SAL)	TGA GCA CAC AGG TCG ACG TCC TAT GWG CTG ACT CAG CCA CC	110
OL4S (HuVL3B-SAL)	TGA GCA CAC AGG TCG ACG TCT TCT GAG CTG ACT CAG GAC CC	111
OL5S (HuVL4B-SAL)	TGA GCA CAC AGG TCG ACG CAG CYT GTG CTG ACT CAA TC	112
OL6S (HuVL5-SAL)	TGA GCA CAC AGG TCG ACG CAG GCT GTG CTG ACT CAG CCG TC	113
OL7S (HuVL6-SAL)	TGA GCA CAC AGG TCG ACG AAT TTT ATG CTG ACT CAG CCC CA	114
OL8S (HuVL7/8-SAL)	TGA GCA CAC AGG TCG ACG CAG RCT GTG GTG ACY CAG GAG CC	115
OL9S (HuVL9-SAL)#	TGA GCA CAC AGG TCG ACG CWG CCT GTG CTG ACT CAG CCM CC	116
OL9S (HuVL10-SAL)#	TGA GCA CAC AGG TCG ACG CAG GCA GGG CTG ACT CAG	117
OJL1 (HuJL1-NOT)	GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT GAC CTT GGT CCC	118
OJL2 (HuJL2/3-NOT)	GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT CAG CTT GGT CCC	119
OJL3 (HuJL7-NOT)	GAG TCA TTC TCG ACT TGC GGC CGC ACC GAG GAC GGT CAG CTG GGT GCC	120

OH1S (HuVH1B-SFI)+	GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG RTG CAG CTG GTG CAR TCT GG	121
OH1S (HuVH1C-SFI)+	GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC SAG GTC CAG CTG GTR CAG TCT GG	122
OH2S (HuVH2B-SFI)	GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG RTC ACC TTG AAG GAG TCT GG	123
OH3S (HuVH3A-SFI)	GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG	124
OH4S (HuVH3C-SFI)	GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG WCY GG	125
OH5S (HuVH4B-SFI)	GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTA CAG CAG TGG GG	126
OH6S (HuVH4C-SFI)	GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG STG CAG CTG CAG GAG TCS GG	127
OH7S (HuVH6A-SFI)	GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTA CAG CTG CAG CAG TCA GG	128
OJH1 (HuJH1/2-XHO)	GAG TCA TTC TCG ACT CGA GAC RGT GAC CAG GGT GCC	129
OJH2 (HuJH3-XHO)	GAG TCA TTC TCG ACT CGA GAC GGT GAC CAT TGT CCC	130
OJH3 (HuJH4/5-XHO)	GAG TCA TTC TCG ACT CGA GAC GGT GAC CAG GGT TCC	131
OJH4 (HuJH6-XHO)	GAG TCA TTC TCG ACT CGA GAC GGT GAC CGT GGT CCC	132

* Mix in 1:1:1 ratio
# Mix in 1:1 ratio
+ Mix in 1:1 ratio

Table 3. Second round VL regions amplification overview

Template	5′ primer	3′ primer	Product	Share in PK/PL(%)	Pool	Share in VL (%)
K1	OK1S	OJK1	K1J1	25	PK1	30
	OK1S	OJK2	K1J2	25		
	OK1S	OJK3	K1J3	10		
	OK1S	OJK4	K1J4	25		
	OK1S	OJK5	K1J5	15		
K2	OK2S	OJK1	K2J1	25	PK2	4
	OK2S	OJK2	K2J2	25		
	OK2S	OJK3	K2J3	10		
	OK2S	OJK4	K2J4	25		
	OK2S	OJK5	K2J5	15		
K3	OK3S	OJK1	K3J1	25	PK3	1
	OK3S	OJK2	K3J2	25		
	OK3S	OJK3	K3J3	10		
	OK3S	OJK4	K3J4	25		
	OK3S	OJK5	K3J5	15		
K4	OK4S	OJK1	K4J1	25	PK4	19
	OK4S	OJK2	K4J2	25		
	OK4S	OJK3	K4J3	10		
	OK4S	OJK4	K4J4	25		
	OK4S	OJK5	K4J5	15		
	OK5S	OJK1	K5J1	25		

K5	OK5S	OJK2	K5J2	25	PK5	1
	OK5S	OJK3	K5J3	10		
	OK5S	OJK4	K5J4	25		
	OK5S	OJK5	K5J5	15		
K6	OK6S	OJK1	K6J1	25	PK6	5
	OK6S	OJK2	K6J2	25		
	OK6S	OJK3	K6J3	10		
	OK6S	OJK4	K6J4	25		
	OK6S	OJK5	K6J5	15		
L1	OL1S	OJL1	L1J1	30	PL1	14
	OL1S	OJL2	L1J2	60		
	OL1S	OJL3	L1J3	10		
L2	OL2S	OJL1	L2J1	30	PL2	10
	OL2S	OJL2	L2J2	60		
	OL2S	OJL3	L2J3	10		
L3	OL3S	OJL1	L3J1	30	PL3	10
	OL3S	OJL2	L3J2	60		
	OL3S	OJL3	L3J3	10		
L4	OL4S	OJL1	L4J1	30	PL4	1
	OL4S	OJL2	L4J2	60		
	OL4S	OJL3	L4J3	10		
L5	OL5S	OJL1	L5J1	30	PL5	1
	OL5S	OJL2	L5J2	60		
	OL5S	OJL3	L5J3	10		

L6	OL6S	OJL1	L6J1	30	PL6	1
	OL6S	OJL2	L6J2	60		
	OL6S	OJL3	L6J3	10		
L7	OL7S	OJL1	L7J1	30	PL7	1
	OL7S	OJL2	L7J2	60		
	OL7S	OJL3	L7J3	10		
L8	OL8S	OJL1	L8J1	30	PL8	1
	OL8S	OJL2	L8J2	60		
	OL8S	OJL3	L8J3	10		
L9	OL9S	OJL1	L9J1	30	PL9	1
	OL9S	OJL2	L9J2	60		
	OL9S	OJL3	L9J3	10		
					VL	100%

Table 4. Second round VH regions amplification overview

Template	5′ primer	3′ primer	Product	Share in PK/PL (%)	Pool	Share in VH (%)
H1	OH1S	OJH1	H1J1	10	PH1	25
	OH1S	OJH2	H1J2	10		
	OH1S	OJH3	H1J3	60		
	OH1S	OJH4	H1J4	20		
H2	OH2S	OJH1	H2J1	10	PH2	2
	OH2S	OJH2	H2J2	10		
	OH2S	OJH3	H2J3	60		
	OH2S	OJH4	H2J4	20		
H3	OH3S	OJH1	H3J1	10	PH3	25
	OH3S	OJH2	H3J2	10		
	OH3S	OJH3	H3J3	60		
	OH3S	OJH4	H3J4	20		
H4	OH4S	OJH1	H4J1	10	PH4	25
	OH4S	OJH2	H4J2	10		
	OH4S	OJH3	H4J3	60		
	OH4S	OJH4	H4J4	20		
H5	OH5S	OJH1	H5J1	10	PH5	2
	OH5S	OJH2	H5J2	10		
	OH5S	OJH3	H5J3	60		

	OH5S	OJH4	H5J4	20		
H6	OH6S	OJH1	H6J1	10	PH6	20
	OH6S	OJH2	H6J2	10		
	OH6S	OJH3	H6J3	60		
	OH6S	OJH4	H6J4	20		
H7	OH7S	OJH1	H7J1	10	PH7	1
	OH7S	OJH2	H7J2	10		
	OH7S	OJH3	H7J3	60		
	OH7S	OJH4	H7J4	20		
					VH	100%

Table 5: Cross-binding activity of PEG/NACl-precipitated and filter-sterilized single-chain phage antibodies to HA of different subtypes, as measured by ELISA. + = binding (>4x background); +/- = low binding (2-4x background) - = no detectable binding; H1= HA of influenza A H1 subtype; H3= HA of influenza A H3 subtype; H5=HA of influenza A H5 subtype; H7=HA of influenza A H7 subtype; B= HA of influenza virus B; Rabies=Glycoprotein of Rabies virus (negative control).

	Phage midi Elisa					
	H1	H3	H5	H7	B	Rabies
sc09-003	+	+	+	+	+	-
sc09-004	+	+	+	+	+	-
sc09-005	+	+	+	+	+	-
sc09-006	+	+	+	+	+	-
sc09-007	+	+/-	+	+	+/-	-
sc09-008	+	+/-	+	+	+/-	-
sc09-009	+	+/-	+	+	+/-	-
sc09-010	+	+	+	+	+/-	-
sc09-011	+	+	+	+	+	-
sc09-012	+	+	+	+	-	-
sc09-029	+	+/-	+	+	-	-
sc09-030	+	+	+	+	+	-
sc09-031	+	+/-	+	+	-	-
sc09-112	+	+	+	+	+	-
sc09-113	+	+	+	+	+	-
sc09-114	+	+	+	+	+	-

Table 6. FACS analysis of PEG/NACl-precipitated and filter-sterilized phage antibodies. + = binding (>4x background); +/- = low binding (2-4x background) - = no detectable binding; PER.C6=untransfected PER.C6 cells (control); mH1, mH3, mH7= membrane bound HA of the subtypes H1, H3 and H7 subtypes, respectively.

	Phage midi Facs (% gated UL)			
	PerC6	mH1	mH3	mH7
sc09-003	-	+	+	+
sc09-004	-	+	+	+
sc09-005	-	+	+	+
sc09-006	-	+	+	+
sc09-007	-	+	+/-	+
sc09-008	-	+	+/-	+
sc09-009	-	+	+/-	+
sc09-010	-	+	+	+
sc09-011	-	+	+	+
sc09-012	-	+	+	+
sc09-029	-	+	-	+/-
sc09-030	-	+	+	+
sc09-031	-	+	-	+/-
sc09-112	-	+	+	+
sc09-113	-	+	+	+
sc09-114	-	+	+	+

Table 7. Data of the CDR regions of the HA specific immunoglobulins. The SEQ ID NO is given between brackets.

IgG#	VH	HC CDR1	HC CDR2	HC CDR3	VL	LC CDR1	LC CDR2	LC CDR3
CR9003	IGHV1-69*06	GGTSNNFG (133)	ISPIFGST (134)	ARHGNYYFYSGMDL (135)	IGLV3-21*02	NVGSNS (136)	DDR (137)	QVWDSSSDHRV (138)
CR9004	IGHV1-69*06	GGTSNNYA (139)	VSPIFGST (140)	ARHGNYYYNSGMDV (141)	IGLV1-44*01	DSNIGRRS (142)	SND (143)	AAWDDSLKGAV (144)
CR9005	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYYSGMDL (145)	IGLV2-14*01	SSDVGGYNY (146)	DVS (174)	CSYAGSAKGV (147)
CR9006	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYYSGMDL (145)	IGLV3-21*02	NIGSKT (148)	GDS (149)	QVWDSSSDHPGAV (150)
CR9007	IGHV1-69*06	GGTSNNYA (139)	ISPIFGSA (151)	ARHGNYYYYSGMDV (152)	IGLV1-44*01	SSNIGSNT (153)	GDD (154)	ATWDDSLNGHV (155)
CR9008	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYYSGMDV (152)	IGLV3-21*02	NIGSKT (148)	GDS (149)	QVWDSSSDHPGAV (150)
CR9009	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYYSGMDV (152)	IGKV1-12*01	QHISSW (156)	SAS (157)	QQANSFPLT (158)
CR9010	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYYSGMDV (152)	IGLV3-21*02	NIGSKT (148)	VDS (159)	QVWDSNSDHPGAV (160)
CR9011	IGHV1-69*06	GGTSNNYA (139)	ISPIFGSA (151)	ARHGNYYYYSGTDV (161)	IGLV1-44*01	DSNIGRRS (142)	SND (143)	AAWDDSLKGAV (144)
CR9012	IGHV1-69*06	GGTSNNYA (139)	ISPIFGSA (151)	ARHGTYYYYSGMDV (162)	IGLV1-40*02	SSNIGAGYD (163)	GNN (164)	QSYDQNLSEGV (165)
CR9029	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYYSGMDV (152)	IGKV3-20*01	QSVSSY (166)	GAS (167)	QQYGSSPFA (168)
CR9030	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYYSGMDV (152)	IGLV3-21*02	NIGSKS (169)	GDS (149)	QVWDSSSDHPGAV (150)
CR9031	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYNSGMDV (141)	IGLV1-40*01	SSNIGAGYD (163)	DNN (169)	QSYDSGLSASPYV (170)
CR9112	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYYSGMDV (152)	IGLV1-40*01	SANIGAGYD (171)	GNN (164)	QSYDSSLSGAL (172)
CR9113	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYYSGMDL (145)	IGLV1-44*01	DSNIGRRS (142)	SND (143)	AAWDASLSGPV (173)
CR9114	IGHV1-69*06	GGTSNNYA (139)	ISPIFGST (134)	ARHGNYYYYSGMDV (152)	IGLV1-44*01	DSNIGRRS (142)	SND (143)	AAWDDSLKGAV (144)

Table 8. Identity cross-tables of the amino acid sequences of the heavy and light chain variable domains.

A.

		Amino acid differences in Heavy Chain															
		SC09-007	SC09-011	SC09-112	SC09-010	SC09-029	SC09-008	SC09-030	SC09-114	SC09-009	SC09-004	SC09-031	SC09-005	SC09-006	SC09-012	SC09-113	SC09-003
Percentage identity	SC09-007		2	3	5	5	6	5	4	5	5	6	7	9	3	11	15
	SC09-011	98.4		5	5	5	6	7	6	7	7	8	9	9	3	13	15
	SC09-112	97.5	95.9		2	2	3	2	1	2	2	3	4	6	6	8	12
	SC09-010	95.9	95.9	98.4		0	3	4	3	4	4	5	6	4	6	10	10
	SC09-029	95.9	95.9	98.4	100.0		3	4	3	4	4	5	6	4	6	10	10
	SC09-008	95.0	95.0	97.5	97.5	97.5		3	2	3	5	6	5	5	7	9	11
	SC09-030	95.9	94.2	98.4	96.7	96.7	97.5		1	2	4	5	4	6	8	6	12
	SC09-114	96.7	95.0	99.2	97.5	97.5	98.4	99.2		1	3	4	3	5	7	7	11
	SC09-009	95.9	94.2	98.4	96.7	96.7	97.5	98.4	99.2		4	5	4	6	8	8	12
	SC09-004	95.9	94.2	98.4	96.7	96.7	95.9	96.7	97.5	96.7		3	6	8	8	10	14
	SC09-031	95.0	93.4	97.5	95.9	95.9	95.0	95.9	96.7	95.9	97.5		5	7	9	11	15
	SC09-005	94.2	92.6	96.7	95.0	95.0	95.9	96.7	97.5	96.7	95.0	95.9		2	8	6	10
	SC09-006	92.6	92.6	95.0	96.7	96.7	95.9	95.0	95.9	95.0	93.4	94.2	98.4		8	8	8
	SC09-012	97.5	97.5	95.0	95.0	95.0	94.2	93.4	94.2	93.4	93.4	92.6	93.4	93.4		12	14
	SC09-113	90.9	89.3	93.4	91.7	91.7	92.6	95.0	94.2	93.4	91.7	90.9	95.0	93.4	90.1		8
	SC09-003	87.6	87.6	90.1	91.7	91.7	90.9	90.1	90.9	90.1	88.4	87.6	91.7	93.4	88.4	93.4	

B.

Percentage identity	Amino acid differences in Light Chain															
	SC09-011	SC09-114	SC09-004	SC09-113	SC09-007	SC09-012	SC09-112	SC09-031	SC09-005	SC09-006	SC09-008	SC09-030	SC09-010	SC09-003	SC09-009	SC09-029
SC09-011		0	2	7	14	29	26	34	44	47	47	45	52	47	62	64
SC09-114	100.0		2	7	14	29	26	34	44	47	47	45	52	47	62	64
SC09-004	98.2	98.2		5	16	27	24	32	42	49	49	47	54	49	62	64
SC09-113	93.6	93.6	95.5		17	25	22	29	41	46	46	44	51	47	62	64
SC09-007	87.3	87.3	85.5	84.6		26	25	32	42	41	41	41	47	43	61	61
SC09-012	73.9	73.9	75.7	77.5	76.6		9	13	39	48	48	47	52	48	61	62
SC09-112	76.6	76.6	78.4	80.2	77.5	91.9		13	37	45	45	44	51	45	60	60
SC09-031	69.9	69.9	71.7	74.3	71.7	88.5	88.5		37	50	50	49	53	46	60	62
SC09-005	60.4	60.4	62.2	63.1	62.2	64.9	66.7	67.3		55	55	54	56	46	64	63
SC09-006	58.0	58.0	56.3	58.9	63.4	57.5	60.2	55.8	51.3		0	3	7	17	64	61
SC09-008	58.0	58.0	56.3	58.9	63.4	57.5	60.2	55.8	51.3	100.0		3	7	17	64	61
SC09-030	59.8	59.8	58.0	60.7	63.4	58.4	61.1	56.6	52.2	97.3	97.3		10	14	62	59
SC09-010	53.6	53.6	51.8	54.5	58.0	54.0	54.9	53.1	50.4	93.6	93.6	90.9		22	67	67
SC09-003	57.7	57.7	55.9	57.7	61.3	57.1	59.8	59.3	58.6	84.6	84.6	87.3	80.0		62	56
SC09-009	45.1	45.1	45.1	45.1	46.0	46.5	47.4	47.4	43.4	42.9	42.9	44.6	40.2	44.1		34
SC09-029	43.4	43.4	43.4	43.4	46.0	45.6	47.4	45.6	44.3	45.5	45.5	47.3	40.2	49.6	68.2	

Table 9. Cross-binding reactivity of IgGs, as measured by ELISA and FACS. H1=soluble recombinant A/New Caledonia/20/1999 H1 HA; H3= soluble recombinant A/Wisconsin/67/2005 H3 HA; H5= soluble recombinant A/Vietnam/1203/04 H5 HA; H7= soluble recombinant A/Netherlands/219/2003 H7 HA; H9= soluble recombinant A/Hong Kong/1073/99 H9 HA; B= soluble recombinant B/Ohio/01/05 influenza B HA; Rabies= rabies glycoprotein; PER.C6=untransfected PER.C6 cells (control); mH1=PER.C6 expressed A/New Caledonia/20/1999 H1 HA; mH3= PER.C6 expressed A/Wisconsin/67/2005 H3 HA; mH7= PER.C6 expressed A/Netherlands/219/2003 H7 HA; ND=not done. + = binding (>10x background); +/- = low binding (2-10x background) - = no detectable binding.

	IgG Elisa							IgG Facs			
	H1	H3	H5	H7	H9	B	Rabies	PerC6	mH1	mH3	mH7
CR9005	+	+	+	+	+	+	-	-	+	+	+
CR9030	+	+	+	+	+	+/-	-	-	+	+	+
CR9112	+	+	+	+	+	+	-	-	+	+	+
CR9113	+	+	+	+	+	+	-	-	+	+	+
CR9114	+	+	+	+	+	+	-	-	+	+	+
CR4098	-	-	-	-	-	-	+	-	-	-	-

Table 10. Cross-neutralizing activity of IgGs; Titers (indicated in µg/ml) are geomean IC50 values as determined according to the Spearman-Karber method of at least duplicate experiments; >100 = not neutralizing at highest tested concentration (100 µg/ml).

	Subtype	Strain	**CR9005**	**CR9112**	**CR9113**	**CR9114**
Group I	H1	A/WSN/33	1.1	0.9	1.1	1.1
		A/New Caledonia/20/99	2.6	1.9	4.4	3.7
		A/Solomon Islands/3/2006	1.4	1.3	2.2	1.8
		A/Brisbane/59/2007	3.4	2	3.1	2.6
		A/California/7/2009	0.7	0.5	0.3	0.3
	H2	A/Env/MPU3156/05	8.8	6.3	8.8	8.8
	H5	A/Hong Kong/156/97	0.8	0.7	0.9	0.4
		A/EW/MPF461/07	10.5	10.5	8.8	10.5
	H6	A/EW/MPD411/07	29.7	10.5	17.7	10.5
	H8	A/EW/MPH571/08	8.8	8.8	8.8	8.8
	H9	A/Hong Kong/1073/99	6.3	3.7	3.7	4.4
		A/Ck/HK/SSP176/09	4.4	4.4	6.3	6.3
Group II	H3	A/Hong Kong/1/68	42	27.6	22.3	19
		A/Johannesburg/33/94	17.7	13.8	32.4	21.9
		A/Panama/2007/1999	28.2	47.5	47.5	39.9
		A/Hiroshima/52/2005	22.9	10.5	13.6	12.5
		A/Wisconsin/67/2005	35.4	29.7	35.4	32.4
		A/Brisbane/10/2007	11.2	5.6	9.4	5.6
	H4	A/WF/MPA 892/06	1.2	0.8	1.3	0.8
	H7	A/Mallard/Netherlands/12/2000	9.6	6.3	6.3	4.8
		A/New York/107/2003	> 100	> 100	> 100	> 100
	H10	A/Chick/Germany/N/49	29.6	26.5	19.8	15.7
	H14	A/Mallard/Astrakhan/263/1982	> 100	> 100	> 100	> 100

REFERENCES

Air MA (1981), Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus. Proc Natl Acad Sci USA 78(12):7639-7643.

De Kruif J et al. (1995), Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Proc Natl Acad Sci USA 92:3938.

Ferguson et al., (2003), Nature 422:428-443.

Fouchier AM et al. (2005), Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79(5):2814-2822.

The World Health Organization Global Influenza Program Surveillance Network (2005), Evolution of H5N1 Avian Influenza Viruses in Asia. Emerg Infect Dis 11:1515-1521.

CLAIMS

1. An isolated antibody or antigen-binding fragment thereof, capable of specifically binding to an epitope in the stem region of the hemagglutinin protein (HA) of influenza A virus subtypes of phylogenetic group 1 and influenza A virus subtypes of phylogenetic group 2 subtypes, and capable of neutralizing at least one or more group 1 influenza A virus subtypes, selected from the group consisting of influenza A viruses comprising HA of the H1, H2, H5, H6, H8, H9 and H11 subtype, and at least one or more group 2 influenza A virus subtypes, selected from the group consisting of influenza A viruses comprising HA of the H3, H4, H7, and H10 subtype, characterized in that the antibody or antigen-binding fragment thereof is also capable of specifically binding to the hemagglutinin protein (HA) of influenza B virus subtypes.

2. An antibody or antigen-binding fragment thereof according to claim 1, wherein the antibody or antigen-binding fragment thereof has no hemagglutination inhibiting activity.

3. An antibody or antigen-binding fragment thereof according to any of the preceding claims, wherein the antibody or antigen-binding fragment thereof is selected from the group consisting of:

an antibody or antigen-binding fragment thereof comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:145, and a light chain CDR1 region of SEQ ID NO: 146, a light chain CDR2 region of SEQ ID NO: 174, and a light chain CDR3 region of SEQ ID NO: 147,

a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:145, and a light chain CDR1 region of SEQ ID NO: 148, a light chain CDR2 region of SEQ ID NO: 149, and a light chain CDR3 region of SEQ ID NO: 150,

a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:145, and a light chain CDR1 region of SEQ ID NO: 142, a light chain CDR2 region of SEQ ID NO: 143, and a light chain CDR3 region of SEQ ID NO: 173;

an antibody or antigen-binding fragment thereof comprising a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, and a light chain CDR1 region of SEQ ID NO: 148, a light chain CDR2 region of SEQ ID NO: 149, and a light chain CDR3 region of SEQ ID NO: 150,

a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, and a light chain CDR1 region of SEQ ID NO: 156, a light chain CDR2 region of SEQ ID NO: 157, and a light chain CDR3 region of SEQ ID NO: 158,

a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, and a light chain CDR1 region of SEQ ID NO: 171, a light chain CDR2 region of SEQ ID NO: 164, and a light chain CDR3 region of SEQ ID NO: 172, and

a heavy chain CDR1 region of SEQ ID NO:139, a heavy chain CDR2 region of SEQ ID NO:134, and a heavy chain CDR3 region of SEQ ID NO:152, and a light chain CDR1 region of SEQ ID

NO: 142, a light chain CDR2 region of SEQ ID NO: 143, and a light chain CDR3 region of SEQ ID NO: 144.

4. A nucleic acid molecule encoding an antibody or antigen-binding fragment thereof according to any one of claims 1-3.

5. An antibody or antigen-binding fragment thereof according to any one of claims 1-3, and/or a nucleic acid molecule according to claim 4, for use as a medicament

6. An antibody or antigen-binding fragment thereof according to claim 5 for use in the prophylactic and/or thereapeutic treatment of influenze infection.

7. A pharmaceutical composition comprising an antibody or antigen-binding fragment thereof according to any one of claims 1-3, and/or a nucleic acid molecule according to claim 4, and a pharmaceutically acceptable excipient.