Title: RECOMBINANT ANTI-MUC1 ANTIBODIES

Abstract: Antibodies having a high specificity and high affinity towards the human gene product MUC1, which in specific variants is associated with neoplastic cells, i.e. tumor tissue, to the use of the antibodies for medical purposes, i.e. for tumor treatment, and for analytical purposes, e.g. for tumor diagnosis. Compositions comprising the antibodies, e.g. for use in the analytical processes and analyses, to pharmaceutical compositions comprising the antibodies, and to use of the antibodies in the manufacture of pharmaceutical compositions for tumor treatment or for diagnostic purposes.
The present invention relates to antibodies having a high specificity and high affinity towards the human gene product MUC1, which in specific variants is associated with neoplastic cells, i.e. tumor tissue, to the use of the antibodies for medical purposes, i.e. for tumor treatment, and for analytical purposes, e.g. for tumor diagnosis. Further, the invention relates to compositions comprising the antibodies, e.g. for use in the analytical processes and analyses, to pharmaceutical compositions comprising the antibodies, and to use of the antibodies in the manufacture of pharmaceutical compositions for tumor treatment or for diagnostic purposes.

Herein, the term antibody is used interchangeably with binding peptide or antigen specific peptide, as the invention provides amino acid sequences as well as nucleic acid sequences encoding the amino acid sequences, which amino acid sequences comprise at least two regions having affinity for one another for association, the amino acid sequence regions forming a paratope with high specificity and high affinity to the tumor associated variant of the MUC1 antigen. The antibodies of the invention can be single chain peptides or one or more associated peptides, forming a paratope with high specificity to an epitope in the tumor-associated variant of the MUC1 protein.

Antibodies of the invention comprise at least two binding peptides forming a paratope with high specificity for MUC1, which binding peptides can be comprised in natural or synthetic peptides, in one or more associated polypeptide chains, e.g. in a single chain variable fragments (scFv), single chain antibody fragments (scFab), in polypeptides comprising one or more natural or synthetic antibody domains, e.g. CH1, CH2, CH3, scFv-Fc and other synthetic antibody fusion proteins, or synthetic association domains, like knob-in-hole providing peptide sections, leucin zipper sections, amphipathic helices, a biotin acceptor domain and an associable avidin or streptavidin domain, each association domain independently contained in a single chain polypeptide or in two or more associated polypeptide chains. Optionally, the antibodies of the invention can comprise at least one effector domain, e.g. selected from a toxin, a radionuclide, a toxic enzyme, a cytotoxic compound, and/or a detectable label, for antigen-specific delivery to tumor cells. Preferably, an IgG, IgA, IgD, or IgE is provided containing the binding peptide of the invention, most preferably the light chain domains of the binding peptide as part of the light chain, and the
heavy chain domains of the binding peptide as part of the heavy chain. In embodiments of Ig containing the binding peptides of the invention, the Fc-portion serves as an effector domain, e.g. for use of the Ig in the production of pharmaceutical compositions for medical use.

5 State of the art

It is known that tumor cells can have a different expression pattern of membrane-bound proteins having extracellular domains, which differences can be used for the specific detection of tumor cells, including the specific delivery of detectable labels or effector compounds to tumor cells, using the specific affinity of antibodies or antibody domains. For example, EP 1366161 B1 describes a polypeptide sequence which is an extracellular domain of a membrane-bound peptide, the peptide participating in the regulation of the immune response.

EP 0941344 B1 describes a humanized antibody specific for the cellular receptor CD1 Ia, the antibody being useful for masking the cell surface receptor.

US 7,368,250 B2 claims a method for diagnosis of cancer cells by analysing the relative expression level of tumor associated epitopes using epitope specific antibodies.

EP 1189931 D1 describes amino acid sections for antibodies that form a specific paratope having affinity to the tumor associated cell surface bound receptor protein pl85HER2. The amino acid sequence having specificity for the tumor associated variant of the receptor is preferably contained in a peptide chain forming an immunoglobulin domain, preferably further associated with a multimerisation domain for increasing the avidity.

Zotter et al. (Cancer Reviews, vol. 11/12, 55 - 101 (1988) describe epithelial MUC1 (also termed CD227, CA-15-3 and PEM) as a transmembrane protein, the O-glycosylation of which is significantly altered in tumor cells. MUC1 in its extracellular domain comprises about 20 to 120 repeating units (variable number of tandem repeats, VNTR). One repeating unit has the following amino acid sequence: TSAPDTRPAHGTV, wherein the potential O-glycosylated amino acids are underlined. Zotter et al. and Hilkens et al. (Cancer Res. 46 (5), 2582 - 2587 (1986)) found that MUC1 is over-expressed in tumor cells and presented over the entire cell surface, whereas in non-neoplastic epithelial cells, MUC1 is expressed only apically. Danielczyk et al. (Cancer Immunol. Immunother. 55 (U), 1337 - 1347 (2006)
describe that due to the presentation of MUC1 also on the non-apical cell surface, MUC1 is accessible by systemically administered antibody, and describe the monoclonal antibody PankoMab having specificity for the tumor variant of MUC1. Murine antibody PankoMab is directed against a glycosylation-dependent epitope of tumor associated MUC1 with an apparent affinity of up to $9 \times 10^{-10}$ M for tumor specific MUC1 (Danielczyk et al., Cancer Immunol. Immunother., 1337-1347 (2006)).

Henderikx et al. (Cancer Res. 58 (19) 4324 - 4332 (1989)) describe single chain Fv antibodies to MUC1 core peptide with an apparent affinity of $8.7 \times 10^{-9}$ M (monovalent affinity $1.4 \times 10^{-7}$ M) for synthetic MUC1 peptide (Henderickx et al., Am. J. Path. (5), 1597 - 1608 (2002)). For optimization and isolation of antibody sections participating in the formation of the paratope, affinity selection using phage display libraries was employed.

From Toleikis (doctoral thesis, Universitat Heidelberg (2004)) a recombinant single chain Fv antibody fragment is known that was isolated from an antibody gene library generated from DNA isolated from patients that were immunized with synthetic MUC1 peptide administered for therapeutical purposes for treatment of metastasing breast cancer. For enriching anti-MUC1 antibodies, a phage display library was generated from the antibody gene library, an affinity selection was performed, alternatingly on synthetic MUC1 peptide and on natural MUC1 isolated from T47D tumor cells. The isolated antibody fragment could be shown by surface plasmon resonance to have an affinity of $2.3 \times 10^{-7}$ M to the glycopeptide, and in immune stains to react specifically with more than 80% of tissue samples of breast cancer (Toleikis et al., (IBCs 17th Annual International Conference Antibody Engineering, December 2006, San Diego, poster presentation).

Objects of the invention

It is an object of the present invention to provide an alternative binding protein, preferably suitable for being comprised in a synthetic or natural antibody, having an improved specificity and/or an improved affinity to a tumor associated extracellular antigen, preferably to the tumor-specific glycosylation variant of MUC1. Preferably, the binding protein and the antibody comprising said binding protein are suitable for production in mammalian cell culture, or by expression in a micro-organism, e.g. yeast or bacteria. Preferably, an antibody
comprising the binding peptide of the invention has a high long-term stability in pharmaceutical formulations, e.g. at long time incubation at 37°C in solution, in combination with stable binding to the antigen in an aqueous medium, e.g. having a reduced propensity for aggregation in aqueous solution. The desired high affinity, high stability, reduced rate of aggregation preferably is in aqueous compositions for use in diagnostics, in pharmaceutical formulations suitable for administration to a patient, as well as during presence in the body of a patient, e.g. in body fluids such as serum.

General description of the invention

The invention achieves the above-mentioned objects by providing binding peptides and antibodies comprising the binding peptides comprised of an antibody heavy chain variable region comprising the amino acid sequence of CDR1 selected from SEQ ID NO: 1 to SEQ ID NO: 6, and one of the amino acid sequences of CDR2 selected from SEQ ID NO: 7 to Seq.-ID No. 12, and/or the amino acid sequence of CDR3 selected from SEQ ID NO: 13 to Seq.-ID No. 18 and/or a light chain variable region comprising the amino acid sequence of CDR1 selected from SEQ ID NO: 19 to Seq.-ID No. 24 and the amino acid sequence of CDR2 selected from SEQ ID NO: 25 to Seq.-ID No. 30, and/or the amino acid sequence of CDR3 selected from SEQ ID NO: 31 to Seq.-ID No. 36. Preferably, the complementary determining regions (CDR) are arranged, from N-terminus to C-terminus, as CDR1 - CDR2 - CDR3, with framework regions interspersed between the CDRs, and preferably with CDRs all of heavy chain variable regions with heavy chain framework regions, or framework regions of light chain variable regions for light chain CDRs, respectively.

More preferably, the CDRs are arranged with interspersed amino acid sequences as FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4, wherein independently, each framework region (FR) can be heavy chain or light chain variable region, but preferably each framework region is of heavy chain variable region for CDRs of heavy chain variable region, whereas FRs for light chain variable region preferably are arranged adjacent light chain variable region CDRs.

More preferably, each binding peptide consists of FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4, all of heavy chain variable region or of light chain variable region only, most preferably
with the FRs and CDRs originating from the heavy chain variable region and the light chain variable region of a single exemplary antibody, respectively.

Generally, all arrangements of amino acid regions, of peptides, or amino acid sequences are noted from N-terminus to C-terminus. Further generally, each heavy chain variable fragment can be associated with each light chain variable fragment, e.g. a heavy chain variable fragment from one preferred binding peptide (e.g. one of Seq.-ID Nos. 85 to 90) can be associated in a single chain peptide (e.g. as an scFv) or as one of two or more associated peptide chains (e.g. as an IgG) with a light chain variable fragment of another binding peptide (e.g. one of Seq.-ID Nos. 91 to 96), preferably of the same binding peptide.

Preferably, the binding peptides and the antibodies comprising the binding peptide have high specific affinity for an epitope having the amino acid sequence RPAP. Preferably, the binding affinity is provided by a light chain variable fragment in association with a heavy chain variable fragment consisting of FR1 - CDRI - FR2 - CDR2 - FR3 - CDR3 - FR4 of light chain and heavy chain antibody regions, respectively, most preferably the light chain variable fragment having the antigen binding affinity of an amino acid sequence of one of SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, and SEQ ID NO: 96, and the heavy chain variable fragment having the antigen binding affinity of an amino acid sequence of one of SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, and SEQ ID NO: 90.

Preferably, the binding peptides of the invention comprise heavy chain variable fragment framework regions comprising the amino acid sequence of FR1, e.g. selected from SEQ ID NO: 37 to SEQ ID NO: 42, and of FR2, e.g. selected from SEQ ID NO: 43 to SEQ ID NO: 48, and/or the amino acid sequence of FR3, e.g. selected from SEQ ID NO: 49 to SEQ ID NO: 54, and/or the amino acid sequence of FR4, e.g. selected from SEQ ID NO: 55 to SEQ ID NO: 60, especially for heavy chain CDRs.

Preferably, the binding peptides of the invention comprise light chain variable fragment framework regions comprising the amino acid sequence of FR1, e.g. selected from SEQ ID NO: 61 to SEQ ID NO: 66, and of FR2, e.g. selected from SEQ ID NO: 67 to SEQ ID NO: 72, and/or the amino acid sequence of FR3, e.g. selected from SEQ ID NO: 73 to SEQ ID...
NO: 78, and/or the amino acid sequence of FR4, e.g. selected from SEQ ID NO: 79 to SEQ ID NO: 84, especially for light chain CDRs.

In one embodiment, the binding peptides of the invention consists of a single chain variable fragment of a heavy chain (VH) of FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4 - CHI - linker - variable light chain (VL) of FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4 - CL, optionally being linked at its N-terminus and/or C-terminus to an effector component.

Preferably, the heavy chain variable region has the affinity of at least one of SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, and SEQ ID NO: 90, and the light chain variable region has the affinity of SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, and SEQ ID NO: 96.

The N-terminal position of the CHI domain preferably has the conformation of one of the amino acid sequences SEQ ID NO: 97 to SEQ ID NO: 102 which are C-truncated sections of natural CHI domains, whereas the N-terminal position of the CL domain preferably has the amino acid sequence of one of SEQ ID NO: 109 to SEQ ID NO: 114 which are C-terminally truncated sections of natural CL domains. Preferably, the linker arranged without interspersing amino acids between the C-terminus of the CHI domain and the N-terminus of the VL domain has the conformation of one of the amino acid sequences SEQ ID NO: 103 to SEQ ID NO: 108.

Generally, the conformation of single amino acid sections comprised in the binding peptides of the invention are obtainable by the respective amino acid sequences given, including amino acid sequences containing one, two, three, four or five mutations, e.g. substitutions, insertions and/or exchanges of single amino acids in each respective amino acid section, e.g. in each FR, each CDR, each constant domain and each linker, while essentially maintaining the characteristic properties of the resulting natural or synthetic antibody, especially its affinity and specificity to an epitope, its stability, produceability in micro-organisms, and its reduced rate of agglomeration. Further, the one or more mutation in one of the light chain and heavy chain preferably is a conservative mutation and/or has an amino acid sequence with at least 95% homology or identity to one of SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89 and SEQ ID NO: 90 for the VH, and to one of SEQ ID NO: 91,
SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95 and SEQ ID NO: 96 for the VL.

Most preferably, the binding peptide comprises one of the amino acid sequences constituted by

SEQ ID NO: 85- SEQ ID NO: 97 - SEQ ID NO: 103 - SEQ ID NO: 91 - SEQ ID NO: 109, or
SEQ ID NO: 86- SEQ ID NO: 98 - SEQ ID NO: 104 - SEQ ID NO: 92 - SEQ ID NO: 110, or
SEQ ID NO: 87- SEQ ID NO: 99 - SEQ ID NO: 105 - SEQ ID NO: 93 - SEQ ID NO: 111, or
SEQ ID NO: 88- SEQ ID NO: 100 - SEQ ID NO: 106 - SEQ ID NO: 94 - SEQ ID NO: 112,

or
SEQ ID NO: 89- SEQ ID NO: 101 - SEQ ID NO: 107 - SEQ ID NO: 95 - SEQ ID NO: 113, or
SEQ ID NO: 90- SEQ ID NO: 102 - SEQ ID NO: 108 - SEQ ID NO: 96 - SEQ ID NO: 114,

with optional moieties linked to the N-terminus and/or C-terminus.

One advantageous embodiment of the invention relates to a binding peptide defined above, which binding peptide is a single chain peptide, preferably said single chain peptide being selected from SEQ ID NO: 122 to SEQ ID NO: 124.

The above mentioned single chain peptide is a scFv fragment.

Another advantageous embodiment of the invention relates to a binding peptide as defined above, wherein the light chain variable amino acid sequence is comprised on a first peptide and the heavy chain variable amino acid sequence is comprised on a second peptide, preferably said first peptide being selected from SEQ ID NO: 125 to SEQ ID NO: 130, and said second peptide being selected from SEQ ID NO: 131 to SEQ ID NO: 136.

The above mentioned first peptide can contain, or not, a signal peptide. Therefore, the above mentioned first peptide with signal peptide is selected from SEQ ID NO 125 to SEQ ID NO 127, and said first peptide without signal peptide is selected from SEQ ID NO 128 to SEQ ID NO 130.

The above mentioned second peptide can contain, or not, a signal peptide. Therefore, the above mentioned first peptide with signal peptide is selected from SEQ ID NO 131 to SEQ ID NO 133, and said first peptide without signal peptide is selected from SEQ ID NO 134 to SEQ ID NO 136.

In one other specific embodiment, the binding peptide mentioned above is constituted by a
couple of a light chain and a heavy chain selected from:
- the couple consisting of the light chain amino acid sequence as set forth in SEQ ID NO: 125
  and the heavy chain amino acid sequence as set forth in SEQ ID NO: 131,
- the couple consisting of the light chain amino acid sequence as set forth in SEQ ID NO: 126
  and the heavy chain amino acid sequence as set forth in SEQ ID NO: 132,
- the couple consisting of the light chain amino acid sequence as set forth in SEQ ID NO: 127
  and the heavy chain amino acid sequence as set forth in SEQ ID NO: 133,
- the couple consisting of the light chain amino acid sequence as set forth in SEQ ID NO: 128
  and the heavy chain amino acid sequence as set forth in SEQ ID NO: 134, this binding peptide corresponds to the antibody HT186-B7,
- the couple consisting of the light chain amino acid sequence as set forth in SEQ ID NO: 129
  and the heavy chain amino acid sequence as set forth in SEQ ID NO: 135, this binding peptide corresponds to the antibody HT186-G2,
- the couple consisting of the light chain amino acid sequence as set forth in SEQ ID NO: 130
  and the heavy chain amino acid sequence as set forth in SEQ ID NO: 136, this binding peptide corresponds to the antibody HT186-DI 1.

It is well know in the art that antibodies are constituted by 2 similar or different, pairs of heavy chain and light chain.

In one advantageous embodiment, the invention relates to a binding peptide as defined above which chosen among:
- the antibody HT186-G2 consisting of a dimer of the couple consisting of the light chain amino acid sequence as set forth in SEQ ID NO: 129 and the heavy chain amino acid sequence as set forth in SEQ ID NO: 135,
- the antibody HT186-B7 consisting of a dimer of the couple the light chain amino acid sequence as set forth in SEQ ID NO: 128 and the heavy chain amino acid sequence as set forth in SEQ ID NO: 134, and
- the antibody HT186-DI 1 consisting of a dimer of the light chain amino acid sequence as set forth in SEQ ID NO: 130 and the heavy chain amino acid sequence as set forth in SEQ ID NO: 136.

In an advantageous embodiment, the invention relates to binding peptide or antibody as defined above produced by the rat hybridoma YB2/0 (cell YB2/3HL.P2.G1 1.16Ag.2O, deposited at the American Type Culture Collection under the number ATCC CRL-1662).
The YB2/0 cell line is chosen since it produces antibodies having an enhanced ADCC activity compared to the same antibody produced by CHO cell line. The invention relates to binding peptide or antibody as defined above, produced by any other means known in the art allowing the increase of the ADCC activity of said antibodies or binding peptides.

**Detailed description of the invention**

The invention is now described in greater detail by way of examples with reference to the figures, wherein

Figures 1 A), B) and C) show tables, wherein the amino acid sequences of regions of binding peptides are shown.

Figure 2 shows ELISA results with the X-axis giving the concentration in µg/mL of antibody containing a binding peptide of the invention in a scFv arrangement, and comparative antibody IIB6 and BSA.

Figure 3 shows SDS-PAGE results, stained for total protein, showing purification of antibody from heterologous expression of antibodies in *E. coli* of A) HT186-D1 1, B) HT200-3A-C1, C) HT220-M-D1, D) HT220-M-G8, E) HT186-B7 and F) HT186-G2 of the present invention.

Figure 4 shows surface plasmon resonance analyses of antibodies, namely under A) HT186-D1 1, B) HT200-3A-C1, C) HT220-M-D1, D) HT220-M-G8, E) HT186-B7, and F) HT186-G2, for comparison on the same chip.

Figure 5 shows FACS results of scFv, namely

A1) comparative anti-MUC scFv IIB6 on MCF7 cells, A2) IIB6 on T47D cells, A3) IIB6 on SKOV3 cells, A4) IIB6 on HEK293-T cells,

B1) HT186-D1 1 on MCF7 cells, B2) HT186-D1 1 on T47D cells, B3) HT1 86-D1 1 on SKOV3 cells, B4) HT1 86-D1 1 on HEK293-T cells,
Cl) HT200-3A-C1 on MCF7 cells, C2) HT200-3A-C1 on T47D cells, C3) HT200-3A-C1 on SKOV3 cells, C4) HT200-3A-C1 on HEK293-T cells,
D1) HT220-M-D1 on MCF7 cells, D2) HT220-M-D1 on T47D cells, D3) HT220-M-D1 on SKOV3 cells, D4) HT220-M-D1 on HEK293-T cells,
E1) HT220-M-G8 on MCF7 cells, E2) HT220-M-G8 on T47D cells, E3) HT220-M-G8 on SKOV3 cells, E4) HT220-M-G8 on HEK293-T cells,
F1) HT186-B7 on MCF7 cells, F2) HT186-B7 on T47D cells, F3) HT186-B7 on SKOV3 cells, F4) HT186-B7 on HEK293-T cells,
G1) HT186-G2 on MCF7 cells, G2) HT186-G2 on T47D cells, G3) HT186-G2 on SKOV3 cells, G4) HT186-G2 on HEK293-T cells

Figure 6 shows ELISA data for antibodies examined in the present invention for long-term stability in aqueous solution at 37°C, for A) HT186-D1, B) HT200-3A-C1, C) HT220-M-D1, D) HT220-M-G8, E) HT186-B7, F) HT186-G2, and G) comparative IIB6.

Figure 7 shows size exclusion chromatograms of purified antibody for determination of unspecific aggregation in solution for A) HT186-D1, B) HT200-3A-C1, C) HT220-M-D1, D) HT220-M-G8, E) HT186-B7, F) HT186-G2, and G) comparative IIB6.

Figure 8 represents a SDS-PAGE Coomassie blue-stained gel under reduced and non-reduced conditions. St: protein ladder; 1: R764-hHMFG1, 2: R764-D1, 3: R764-B7, and 4: R764-G2.

Figure 9 represents anti-MUC1 IgG binding curve to the insoluble antigen in the microtitration plate. It corresponds to preliminary results.

Figure 10 represents the titration curve of antibodies B7, D11 and G2 against MUC-I peptide by ELISA.

Figure 11 represents the FACS comparison of the antibodies reactivity with T74D cells. MFI represents the mean fluorescence intensity.
Figure 12 represents the FACS overlay plots with 100 µg/ml of antibody. Black line: detection antibodies only, dashed black: IgGl isotype control antibody, A line: DII IgG, B line: B7 IgG, C line: G2 IgG and D line: hHMFGl IgG.

Figure 13 represents the FACS overlay plots with 31.6 µg/ml of antibody. Black line: detection antibodies only, dashed black: IgGl isotype control antibody, A line: DII IgG, B line: B7 IgG, C line: G2 IgG and D line: hHMFGl IgG.

Figure 14 represents the FACS overlay plots with 10 µg/ml of antibody. Black line: detection antibodies only, dashed black: IgGl isotype control antibody, A line: DII IgG, B line: B7 IgG, C line: G2 IgG and D line: hHMFGl IgG.

Figure 15 represents the FACS overlay plots 3.16 µg/ml of antibody. Black line: detection antibodies only, dashed black: IgGl isotype control antibody, A line: DII IgG, B line: B7 IgG, C line: G2 IgG and D line: hHMFGl IgG.

Figure 16 represents the FACS overlay plots with 1 µg/ml of antibody. Black line: detection antibodies only, dashed black: IgGl isotype control antibody, A line: DII IgG, B line: B7 IgG, C line: G2 IgG and D line: hHMFGl IgG.

Figure 17 represents the FACS overlay plots with 0.316 µg/ml of antibody. Black line: detection antibodies only, dashed black: IgGl isotype control antibody, A line: DII IgG, B line: B7 IgG, C line: G2 IgG and D line: hHMFGl IgG.

Figure 18 represents the FACS overlay plots with 0.1 µg/ml of antibody. Black line: detection antibodies only, dashed black: IgGl isotype control antibody, A line: DII IgG, B line: B7 IgG, C line: G2 IgG and D line: hHMFGl IgG.

Figure 19 represents the FACS overlay plots with 0.032 µg/ml of antibody. Black line: detection antibodies only, dashed black: IgGl isotype control antibody, A line: DII IgG, B line: B7 IgG, C line: G2 IgG and D line: hHMFGl IgG.
Figure 20 represents the FACS overlay plots with 0.01 µg/ml of antibody. Black line: detection antibodies only, dashed black: IgG1 isotype control antibody, A line: D11 IgG, B line: B7 IgG, C line: G2 IgG and D line: hHMFG1 IgG.

Figure 21 represents the FACS overlay plots with 0.003 µg/ml of antibody. Black line: detection antibodies only, dashed black: IgG1 isotype control antibody, A line: D11 IgG, B line: B7 IgG, C line: G2 IgG and D line: hHMFG1 IgG.

Figure 22 represents the ADDC. The graph represents the percentage of cell lysis with respect to the concentration of antibodies.

Figure 23 represents the D11 IgG antibody stability in PBS, human inactivated serum or human serum. The stability is measured by ELISA during 28 days.

Figure 24 represents the D11 scFv Fc fragment stability in PBS, human inactivated serum or human serum. The stability is measured by ELISA during 28 days.

Figure 25 represents the D11 hHMFG1 IgG antibody stability in PBS, human inactivated serum or human serum. The stability is measured by ELISA during 28 days.

Figure 26 the percentage of internalisation after incubation 1 hour at 37°C (white columns) compared with incubation at 4°C (black columns). FGl represents control antibody. Y-axis represents the mean of fluorescence intensity.

In Figure 1, there are shown amino acid sequences for hypervariable complementary determining regions (CDR) CDR1, CDR2, CDR3 in their preferred arrangement between framework regions (FR) FR1, FR2, FR3 and FR4 for the heavy chain variable fragment (VH) and the light chain variable fragment (VL), respectively, for antibodies comprising, preferably constituting, the binding peptides of the invention. The assignment of amino acid sequence sections to hypervariable region sections, namely to FR and CDR, respectively, allows to freely recombine at least one of FR and one of CDR, preferably in the arrangement of FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4, independently from heavy chain and light chain variable regions, to obtain an antibody according to the invention. The preferred antibodies of
the invention comprise CDRs and FRs from the binding peptides designated HT1 86-Dl 1, HT200-3A-C1, HT220-M-D1, HT220-M-G8, HT186-B7 and/or HT186-G2, most preferably the heavy chain variable fragments in association with the light chain variable fragments, e.g. SEQ ID NO: 85 associated with SEQ ID NO: 91, Seq.-ID-No 86 associated with SEQ ID NO: 92, SEQ ID NO: 87 associated with SEQ ID NO: 93, SEQ ID NO: 88 associated with SEQ ID NO: 94, SEQ ID NO: 89 associated with SEQ ID NO: 95, and SEQ ID NO: 90 associated with SEQ ID NO: 96 respectively.

Further, Figure 1 contains amino acid sequences of scFv antibody comprising the binding peptide consisting of VH -truncated CH1 - linker - VL - truncated CL , wherein preferably one antibody comprises the VH and VL region originating from one of HT1 86-Dl 1 (Seq.-ID No. 85 or Seq.-ID No. 91), HT200-3A-C1 (Seq.-ID No. 86 or Seq.-ID No. 92), HT220-M-D1 (Seq.-ID No. 87 or Seq.-ID No. 93), HT220-M-G8 (Seq.-ID No. 88 or Seq.-ID No. 94), HT186-B7 (Seq.-ID No. 89 or Seq.-ID No. 95), or HT186-G2 (Seq.-ID No. 90 or Seq.-ID No. 96). Further, antibodies of the invention also comprise the association of one VH of one of the amino acid sequences SEQ ID NO: 85 to SEQ ID NO: 90 with one VL of one of the amino acid sequences SEQ ID NO: 91 to SEQ ID NO: 96, with one of the CH1 regions of one of the amino acid sequences SEQ ID NO: 97 to SEQ ID NO: 102, one of the linkers of one of the amino acid sequences SEQ ID NO: 103 to SEQ ID NO: 108, and one of the amino acid sequences SEQ ID NO: 103 to SEQ ID NO: 108 for the linker (also termed Yol), and one of the CL of amino acid sequences SEQ ID NO: 109 to SEQ ID NO: 114.

Embodiments according to the invention comprise the binding peptides being linked to effector components, which effector components accordingly can be coupled to the exemplary antibodies shown, e.g. by translation of a conjugate comprising the antibody and the effector component, e.g. by translation from a single coding nucleic acid sequence. Optionally, a nucleic acid sequence can be used for expression, which nucleic acid sequence encodes the antibody of the invention, e.g. as an Ig, and/or including a multimerization domain and/or an effector component.

An scFv antibody section, which can optionally be coupled to an effector component, essentially consists of a light chain variable region connected to a heavy chain variable region via an intermediate linker peptide such that the variable regions can associate to one another.
for forming the antigen binding region, which is also termed paratope. As an alternative embodiment, two associable scFv antibodies are produced, which associate to a diabody, in each case preferably with association of one heavy chain variable region and one light chain variable region contained in one single polypeptide chain or contained in separate polypeptide chains.

An example for heterologous multimerization domains, a helix-turn-helix-motive is a component C- or N-terminally added to a light chain variable region or a heavy chain variable region, allowing the association of two single polypeptide antibodies. In the alternative to a synthetic multimerization domain, natural antibody domains can be contained within the antibody, e.g. connected to a light chain variable region and/or a heavy chain variable region, wherein the antibody domains preferably are selected from a light chain constant region CH1, CH2 and/or CH3, e.g. resulting in the antibody being a Fab or a natural immunoglobulin, preferably IgG.

As further examples for multimerization domains, the biotin acceptor domain (BAD, amino acid sequence available under accession No. 2zta, PDB) is a suitable component linked to an scFv, which BAD (described in Schatz, Biotechnology 11 (10), 1138-1143 (1993)) after biotinylation allows the association of the biotinylated antibody to avidin or streptavidin. Further multimerization domains that can be contained in one polypeptide chain comprising the antibody of the invention are the leucin zipper domain, the modified leucin zipper domain tetraZIP which results in a tetramerisation of antibody containing tetraZIP, or the tetramer relation domain of p53 or amphipathic helices (amino acid sequences available in Plückthun et al., Immunotechnology 3(2), 83-105 (1997)), originating from the human transcription effector suppressor p53 (amino acid sequence available under accession No. 2j8z, PDB). Preferably, linker peptide sequences are arranged between multimerization domains and antibody regions to avoid interference of the multimerization with antigen binding.

Using standard DNA cloning procedures, a library of nucleic acid sequences encoding antibody according to the invention was generated by enriching MUC1-binding VH and VL that were displayed on the surface of phage particles.
For mutation of antibody encoding genes contained in the antibody gene library, amplification using error prone PCR utilizing Mutazymell® DNA polymerase (obtainable from Stratagene, Amsterdam) was used, which statistically generates a mutation rate of up to 16 nucleotide exchanges per 1000 bp in one PCR of approximately 35 amplification cycles, with a mutation rate of A or T to N of approximately 50%, and G or C to N of approximately 44%.

For cloning of antibody comprised of a combination of framework regions and CDRs in a new arrangement, preferably by exchanging one framework region for a framework region of the same localization (as indicated by the same numbering of FR) and/or of a CDR, respectively, PCR cloning could be employed, using PCR primers overlapping the region encoding the desired amino acid exchanges.

The enrichment of phage encoding and expressing antibody sections with increased antigen binding, i.e. having a lower dissociation constant, was obtained by the so-called panning procedure, comprising the step of incubating antibody presenting phage with immobilized antigen, followed by extensive washing in phosphate buffered saline including Tween20 for 7 days in 2 L PBS under slight stirring at 4 °C, followed twice by further washing of recovered immobilized antigen with an additional 1 L PBS at 4 °C for 7 days each. Finally, phages that were bound to immobilized antigen were released by trypsination and used for infection of E. coli. For immobilisation of antigen, microtitre plates were coated with the antigen, or alternatively, suspendable synthetic beads coated with streptavidin were coated with biotinylated antigen. As the antigen, synthetic MUC1 peptide APDTRPAPGSTAPPAC (Seq.-ID No. 115) or APDTRPAPGSTAPPAPGSTA (Seq.-ID No. 116) or Biotin-β-A-β-A-APDTRPAPGSTAPPAGVTSAAPDRPAPGSTA (Seq.-ID No. 117) or APDTRPAPGSTAPPAGVTSAAPDRPAPGSTAC (Seq.-ID No. 118) was used for the analysis of monoclonal soluble antibody fragments.

As a further variation of panning in solution, incubation of the antigen with the antibody presenting phage was in the presence of unlabelled antigen (MUC1 peptide), serving as a competitor for the immobilized MUC1 peptide.

From the various steps of the enrichment of anti-MUC1 antibody presenting phage, the heavy chain variable regions of SEQ ID NO: 85 to SEQ ID NO: 90 were found, associated with light
chain variable regions of amino acid sequences SEQ ID NO: 91 to SEQ ID NO: 96, respectively.

Interestingly, in comparison to anti-MUC1 antibody IIB6, these antibodies show a higher affinity for MUC1 antigen, a drastically increased stability, e.g. in a long time incubation at 37°C in aqueous solution with maintenance of the antigen specificity as determined in an ELISA assay, affinity to an advantageous binding epitope on the MUC1 antigen and a low propensity to agglomerate independent of multimerization domains linked to the binding peptides of the invention. Non-specific agglomeration in solution generally leads to a reduction in biological activity and is therefore undesired. Stability data are shown in Example 5.

Generally, an expression vector for use in the preferred process for production of antibody according to the invention, e.g. for heterologous expression in a micro-organism, preferably in a host bacterium, e.g. a Gram-negative bacterium, contains a nucleic acid sequence encoding the antibody and, preferably contains the coding region for an N-terminal signal sequence for transport of the translation product into the periplasm. As it is known in the art, an expression vector in addition to vector specific elements like an origin of replication and a selection marker, according to the invention contains a nucleic acid sequence encoding the antibody of the invention comprised in an expression cassette, optionally encoding additional effector components, to produce a single chain amino acid sequence comprising the binding peptide. As one example for a nucleic acid sequence, functionally arranged from 5’ to 3’, the coding region for an scFv is contained, optionally followed by a coding sequence for a heavy multimerization domain. The linker peptide connecting the heavy chain variable region binding peptide with the light chain variable region in the embodiment of an scFv as used in the examples has the sequence indicated in Table 1C. In the alternative to this arrangement, the scFv can contain the light chain variable region binding peptide - linker - heavy chain variable region binding peptide.

For production of antibody according to the invention, the scFv embodiment is preferred, for example in addition including a C-terminally added multimerization domain BAD, tetraZIP, ZIP, dHLX, p53, preferably VH for heavy chain binding peptide and VL for light chain binding peptide, respectively, optionally followed by a C-terminally arranged His(6)-tag.
Further, the coding sequence encoding an N-terminally arranged periplasm signal sequence (e.g. pelB) for transport of the antibody into the periplasmic space. The coding sequence can be functionally arranged between a standard bacterial promoter (PA1/04/07) and a T7 terminator, transferred into *E. coli* and cultivated. Multimerization domains dHLX, ZIP, p53 and tetraZIP are self-associating, whereas for antibody containing BAD, biotin ligase is concurrently expressed in *E. coli* to covalently couple biotin to the C-terminus of the polypeptide chain. Biotinylated scFv after expression and purification are incubated in the presence of streptavidin.

Alternatively, the antibodies of the invention could be expressed in animal cell culture from a suitable eukaryotic expression cassette containing the antibody encoding nucleic acid sequence.

Following collection of cells after induction of the bacterial promoter, periplasmic proteins were isolated and affinity purified by metal chelate chromatography using the His-tag of the antibodies. For comparative antibody IIB6 (anti-MUC1, according to Toleikis, loc. cit.) and for antibody according to the invention, the following production efficiencies were determined. A comparison of the yields of antibodies HTI86-DI1, HT200-3A-C1, HT220-M-DI1, HT220-M-G8, HT186-B7, and HT186-G2, also scFv, show comparable production rates and yields.

### Table 1: Production yield (iig/L) of antibody variants in bacterial expression

<table>
<thead>
<tr>
<th>scFv</th>
<th>Conc. in eluate (mg/mL)</th>
<th>Vol. Eluate (mL)</th>
<th>Yield scFv/culture volume (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIB6</td>
<td>1.09</td>
<td>2</td>
<td>&gt;2.2</td>
</tr>
<tr>
<td>HT186-DI1</td>
<td>1.45</td>
<td>2</td>
<td>&gt;2.9</td>
</tr>
<tr>
<td>HT200-3A-C1</td>
<td>0.80</td>
<td>2</td>
<td>&gt;1.6</td>
</tr>
<tr>
<td>HT220-M-DI</td>
<td>0.79</td>
<td>2</td>
<td>&gt;1.6</td>
</tr>
<tr>
<td>HT220-M-G8</td>
<td>1.60</td>
<td>2</td>
<td>&gt;3.2</td>
</tr>
<tr>
<td>HT186-B7</td>
<td>0.49</td>
<td>5</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>HT186-G2</td>
<td>0.42</td>
<td>2</td>
<td>&gt;0.8</td>
</tr>
</tbody>
</table>

SDS-PAGE analytical results from samples of the purification process steps from antigen expressing *E. coli* are shown in Figure 3, indicating under A) preferred antibody HTI 86-DI 1.
B) HT200-3A-C1, C) HT220-M-D1, D) HT220-M-G8, E) HT186-B7 and F) HT186-G2. For these antibodies according to the invention, a prominent band for the elution sample (elution 1) of the molecular size of the scFv (indicated by arrow) is visible.

The surface plasmon resonance analyses shown in Figures 4 A) to F) are for antibody concentrations from 2 nM (lower curve), 5 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM, 100 nM to 200 nM (top curve) on immobilized 47 RU (resonance units) MUC1 peptide (15 aa + cystein, APDTRPAPGSTAPPAC) (SEQ ID NO: 115 with an additional cystein) on Chip 1. Analyses on 14 RU MUC1 peptide (15 aa + cystein, SEQ ID NO: 115 with an additional cystein) on Chip 2 gave similar values. Detailed results are given in the following table 2.

Table 2: MUC - peptide specific affinity of scFv antibodies from surface plasmon resonance measurement

<table>
<thead>
<tr>
<th>Chip 1 – 47 RU MUC1-peptide (15aa Cys)</th>
<th>antibody</th>
<th>$k_a$ (1/Ms)</th>
<th>$k_d$ (1/s)</th>
<th>$R_{max}$ theor.</th>
<th>$K_A$ (1/M)</th>
<th>$K_D$ (M)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIB6</td>
<td>-</td>
<td>-</td>
<td>41</td>
<td>3.2 x 10^6</td>
<td>3.1 x 10^{-7}</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>HT186-D11</td>
<td>3.8 x 10^4</td>
<td>2.1 x 10^{-3}</td>
<td>519</td>
<td>1.8 x 10^9</td>
<td>5.7 x 10^{-10}</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>HT200-3A-C1</td>
<td>4.9 x 10^4</td>
<td>1.0 x 10^{-4}</td>
<td>211</td>
<td>4.8 x 10^8</td>
<td>2.1 x 10^{-9}</td>
<td>4.3</td>
<td></td>
</tr>
</tbody>
</table>
From these results, it can be seen that the antibodies of the invention show a very high affinity to the specific antigen, with an approximate dissociation constant $K_D$ in the range of $2.1 \times 10^{-9}$ to $5.7 \times 10^{-10}$ M, which is a significantly higher affinity than that previously found for comparative antibody IIB6 having a $K_D$ of $3.1 \times 10^{-7}$.

Using immobilized synthetic overlapping peptides for association to the antibodies of the invention, it was found that the epitope recognised by the antibodies has the amino acid sequence RPAP, which is a section of the VNTR region of MUC1. It is assumed that the high affinity of the antibodies of the invention for MUC1 in the tumor-associated O-glycosylation over the non-tumor associated O-glycosylation of MUC1 is caused by the epitope being exposed in the tumor-associated O-glycosylation.

Using flow-cytometry (FACS) wherein the binding peptides of the invention were embodied as scFv fragments of approx. 200 000 cultivated human neoplastic cells (MUC1-positive human adenocarcinoma cell lines T47D and MFC-7, MUC1-positive ovarian carcinoma cell line SKOV3, and MUC1-negative HEK293-T), with immune staining by incubation with antibodies according to the invention, removal of unbound antibody by washing and specific detection by a secondary antibody-dye (mouse anti-His$_6$-IgG, combined with goat anti-mouse-IgG-FITC conjugate) and washing, MUC1 specific detection of cells by antibodies comprising the binding peptides of the invention could be analysed. From the FACS results,
which due to the gate settings of the flow cytometer are shown in Figure 5 for living cells only, it can be seen that the antibodies of the invention HTI 86-D1, HT200-3A-C1, HT220-M-D1, HT220-M-G8, HT186-B7, and HT186-G2 each have a high affinity to surface cell bound, tumor associated MUC1, whereas no significant or only very low non-specific reaction with the control cell line HEK293-T occurred. Comparative clone IIB6 showed a less selective association with MUC1.

For the stability ELISA, the results of which are shown in Figures 6 A) to F), and G) for comparative IIB6, respectively, detection of bound scFv was by mouse anti-His-tag IgG, followed by goat anti-IgG HRP-conjugate, using colorimetric detection of HRP (horse radish peroxidase) with TMB. For negative controls, bovine serum albumin (BSA) was used as the antigen immobilized to the microtiter plate wells.

For stability testing, purified antibodies of the invention were incubated at a concentration of 10 µg/mL at 37°C, with measurement of the relative specificity for MUC1 antigen in an ELISA. Results are shown in Figure 6, indicating that the antibodies of the invention have an improved long-term stability over a period of at least seven days, preferably of at least 30 days, at concentrations from 5 µg/mL (top curve), 1.6 µg/mL, 0.59 µg/mL, 0.16 µg/mL, 0.05 µg/mL, 0.016 µg/mL to 0.005 µg/mL (bottom curve) each, at least superior in relation to comparative antibodies generated during the panning procedure, for which results are shown under A) to F), and G) for comparative IIB6 of Toleikis.

The propensity for forming non-specific aggregates in solution was estimated by size exclusion chromatography on Sephadex (Superdex 200 10/300 GL, using 0.5 mL/min flow rate and UV detection. Results are shown in Figure 7, indicating that the antibodies of the invention in comparison to comparative antibodies showed a significantly reduced proportion of dimers or higher aggregates.

Results are shown in Figure 7 A) to F) for antibodies of the invention, and in G) for the comparative antibody. From the higher proportion of monomer, and reduced proportion of aggregates in the antibodies of the invention when compared to G), it can be concluded that the antibodies of the invention better maintain their structure in solution and therefore an
increased activity over storage and treatment periods can be expected. This result confirms the
stability tests shown in Figures 6 A) to F).

**EXAMPLE 1: anti MUC-I antibodies expressing YB2/0 clones**

Pools of transfected YB2/0 cells stably expressing one of the anti-MUC1 hHMFG1, D11, B7 and G2 were obtained.

At least 10 mg of each antibody was produced by batch agitated culture.

The above mentioned antibodies are possibly used for the treatment of some cancers, for instance lung cancer.

1- Cloning Procedures

a- Cloning of λ constant light chain (λ-CL)

The λ-CL chain was amplified by PCR from the vector CHL558-02, which has been digested by *Apa* I and *Nhe* I restriction enzymes to avoid contamination.

PCR was performed in a volume of 100µL with the two following primers:

- Primer forward 5'-tgctgcaccaagtctcactc-3'  SEQ ID NO: 119,
- Primer reverse 5'-ctctagctctctggatcct-3'  SEQ ID NO: 120.

The components of the reaction are as follows:

- DNA template (CHL558-02 vector) 1µg/mL ............... 1 µL
- PCR reaction Buffer 5x .................................... 20 µL
- 40 mM dNTP (10mM each) ......................... 2 µL
- Primer forward 10pmol/µL.............................. 5 µL
- Primer reverse 10pmol/µL.............................. 5 µL
- Phusion DNA polymerase (4U/µL) .................... 1 µL
- water ................................................. 66 µL

The reaction was carried out according to the following program:

98°C; 30s

98°C; 10s ]

50°C; 20s \ x 29

72°C; 15s J

72°C; 10 min
After PCR, the expected band of 339 base pairs (bp) was obtained.

PCR fragment was purified with NucleoSpin® Extract II Kit (Macherey Nagel), and resuspended in 75µL of Elution buffer.

Purified PCR fragment was:
- First, digested with Dra III restriction enzyme, and purified with NucleoSpin® purification kit, and
- Second, digested with Xba I restriction enzyme, and purified with NucleoSpin® purification kit.

In the same time, the target vector was simultaneously digested with Dra III and Xba I, digestions followed by dephosphorylation of the 5'end by using Shrimp Alcaline Phosphatase (SAP). Vector was purified on agarose gel and with with NucleoSpin® Extract II Kit (Macherey Nagel).

Double digested PCR fragment and vector were then ligated by using T4 DNA ligase (1.5U) in a respective ratio 3:1 and 10:1. As control, vector alone has been treated with T4 DNA ligase.

After incubation (o/n at 16°C), T4 DNA ligase was inactivated at 65°C for 15 min.

Ligation products (Vector insert 1:3 and 1:10, and vector alone) were desalted by ethanol precipitation and resuspended in 40µL of water.

E. coli XL1-Blue MRF’ electrocompetent bacteria were then transformed by electroporation (1.7kV) with 1/4 of one of the ligation products. Immediately after electroporation, cells were resuspended in SOC medium and incubated for 1h at 37°C and plated on 2x YT agar plates containing ampicillin. Plates were incubated o/n at 37°C.

On the plates where bacteria transformed with vector alone were plated, 15 colonies have emerged.

On the plates where bacteria transformed with vector + insert 1:3 were plated, about 50 colonies have emerged (A).

On the plates where bacteria transformed with vector + insert 1:10 were plated, about 100 colonies have emerged (B).
14 colonies from (A) and 14 colonies from (B) were tested by PCR using the following primers:

Primer forward 5'-tggctgcaccaagtgtcactc-3'  SEQ ID NO: 119.

Primer reverse Seq 5'-gggaggggcaaacaacagatggc-3'  SEQ ID NO: 121.

The components of the reaction are as follows:
- Bacteria in medium .................................. 1 µL
- PCR reaction Buffer 10x ................................... 2 µL
- 40 mM dNTP (10mM each) .............................. 1 µL
- Primer forward 10pmol/µL .......................... 0.2 µL
- Primer reverse 10pmol/µL .......................... 0.2 µL
- RED DNA Taq polymerase (4U/µL) .................. 0.3 µL
- water ..................................................... 16.3 µL

The reaction was carried out according to the following program

95°C; 100s
95°C; 60s 1
52°C; 45s \( \frac{1}{2} \times 29 \)
72°C; 45s J
72°C; 10 min
4°C; 5 min and 16°C; \( \infty \).

After separation on 1% agarose gel, all the 28 colonies tested were positive.

4 clones were amplified to prepare maxi DNA preparations, and, said maxi preparations were sequenced by using Primer reverse Seq 5'-gggaggggcaaacaacagatggc-3'  SEQ ID NO: 121.

No mutation was found. The HT327-1 clone was chosen.

**b- Geπaral cloning strategy**

For cloning of anti-MUC1 variable domains the IgG expression vector CHK622-08 (optimized for expression of human IgG1 antibodies) was modified. The light chain kappa constant gene was replaced with the light chain lambda constant gene provided in the vector CHL558-02 as described in section a- Cloning of \( \lambda \) constant light chain (\( \lambda \)-CL).

Anti-MUC1 scFv variable domains were bioinformatically analyzed by using the VBASE2 portal (http://www.vbase2.org).
Synthetic genes of VH and VL genes of the clones HTl 86-D1 1, HT186-B7 and HT186-G2 (including the desired restriction sites and the human signal sequence of the corresponding human germline genes) were ordered at Genscript (GenScript USA Inc. 120 Centennial Ave. Piscataway, NJ 08854 USA). The genes were provided in a pUC57 vector.

The synthetic variable domain genes then were cloned in a two step cloning. First the VL gene of each variant (HT186-D1 1, B7, G2) was cloned into the vector CHK622-08 lambda using Spel and DraIII restriction sites. Subsequently the VH genes of HTl 86-D1 1, B7, G2 were cloned into the vectors of the light chain gene cloning using Nhel and Apal restriction sites. After cloning, DNA sequencing of all vector areas affected by cloning was performed to validate the success of cloning.

The following clones were finally created:

HT335-1-1 → Vector: CHK622-08 lambda → encoding: HTl 86-D1 1 IgG.
HT335-3-2 → Vector: CHK622-08 lambda → encoding: HT186-G2 IgG.
HT337-1-2 → Vector: CHK622-08 lambda → encoding: HT186-B7 IgG.

2- Cell transfection procedure

2.2.1 Host cell line of transformation

The host cell line of transformation, intended to integrate the expression vector and produce the antibody, is the line YB2/0.

YB2/0 cells were received defrosted. They were kept in view of transfections at 2.10^5 cells/mL in EMS medium, 5% dialyzed FBS (table 1).

Table 1: Monitoring of the YB2/0 line before transfection

<table>
<thead>
<tr>
<th>Cell density (10^5 c/mL)</th>
<th>Viability (%)</th>
<th>Transplanting-cell density (10^5 c/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.4</td>
<td>96</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>91</td>
<td>2</td>
</tr>
<tr>
<td>2.8</td>
<td>93</td>
<td>Centrifuged volume: 200 mL</td>
</tr>
</tbody>
</table>

2.2.2 Expression vectors

The expression vectors used for transfection are listed in table 2.

Table 2: Vectors used for transfection

<table>
<thead>
<tr>
<th>Vector</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT186-B7 linearized by EcoRV</td>
<td>1,365 ng/mL</td>
</tr>
<tr>
<td>HT186-D11 linearized by EcoRV</td>
<td>1,456 ng/mL</td>
</tr>
</tbody>
</table>
3. RESULTS

3.1 Electroporation and transfection rates

Electroporation was carried out as follows:
- voltage 230V
- capacitance 960 µF

Six cuvettes were prepared:
- Cuvette 1: 42.7 µg of the vector HT186-B7 linearized by EcoRV
- Cuvette 2: 42.7 µg of the vector HT186-D1 linearized by EcoRV
- Cuvette 3: 42.7 µg of the vector huHMFG1 linearized by NotI
- Cuvette 4: 42.7 µg of the vector HT186-G2 linearized by EcoRV
- Positive control cuvette: 25.2 µg of the linearized vector H416-24
- Negative control cuvette: no vector

After electroporation, cells were distributed from the electroporation cuvettes to cell culture plates as follows:
- Cuvette 1 to 4 (per cuvette): 5 P24 at 25,000 cells/well
  1 P96 at 500 cells/well
  1 P96 at 100 cells/well
- Positive control cuvette T+: 1 P96 at 5,000 cells/well
  1 P96 at 500 cells/well
  1 P96 at 100 cells/well
- Negative control cuvette T-: 1 P96 at 5,000 cells/well
  1 P96 at 500 cells/well
  1 P96 at 100 cells/well

The characteristics of the transfection are listed in table 3.

Table 3: Characteristics of the transfection

<table>
<thead>
<tr>
<th>Cuvette No.</th>
<th>Pulse time (in ms)</th>
<th>Recommendation</th>
<th>Plate</th>
<th>J+3 counting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Living cells</td>
</tr>
</tbody>
</table>
Cells were kept in a selective medium: RPMI medium, 5% dialyzed FBS, 0.5 g/L geneticine, 25 nM MTX for P24 and P96 plates at 5,000 cells/well and RPMI, 5% dialyzed FBS, 1 g/L geneticin for the others in order to determine transfection rates. The medium was renewed every 7 days during 4 weeks for P96 plates and up to the establishment of pools for P24 plates.

Appearance rates of the transformants are shown in table 4.

Table 4: Appearance rates of transformants in 96-well plates

<table>
<thead>
<tr>
<th>Cuvette No</th>
<th>Selection</th>
<th>Cells/well</th>
<th>Number of grown wells/P96</th>
<th>Theoretical number of grown wells/P96</th>
<th>Transfection in accordance</th>
<th>Transfection rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RPMI + 5% dialyzed FBS + G418 1 g/L</td>
<td>100</td>
<td>1</td>
<td>5</td>
<td>Not applicable</td>
<td>1/1870</td>
</tr>
<tr>
<td>1</td>
<td>RPMI + 5% dialyzed FBS + G418 1 g/L</td>
<td>500</td>
<td>1</td>
<td>27</td>
<td>Not applicable</td>
<td>1/1515</td>
</tr>
<tr>
<td>2</td>
<td>RPMI + 5% dialyzed FBS + G418 1 g/L</td>
<td>100</td>
<td>1</td>
<td>10</td>
<td>Not applicable</td>
<td>1/910</td>
</tr>
<tr>
<td>2</td>
<td>RPMI + 5% dialyzed FBS + G418 1 g/L</td>
<td>500</td>
<td>1</td>
<td>34</td>
<td>Not applicable</td>
<td>1/1144</td>
</tr>
<tr>
<td>3</td>
<td>RPMI + 5% dialyzed FBS + G418 1 g/L</td>
<td>100</td>
<td>1</td>
<td>6</td>
<td>Not applicable</td>
<td>1/1550</td>
</tr>
<tr>
<td>3</td>
<td>RPMI + 5% dialyzed FBS + G418 1 g/L</td>
<td>500</td>
<td>1</td>
<td>13</td>
<td>Not applicable</td>
<td>1/3437</td>
</tr>
<tr>
<td>4</td>
<td>RPMI + 5% dialyzed FBS + G418 1 g/L</td>
<td>100</td>
<td>1</td>
<td>9</td>
<td>Not applicable</td>
<td>1/1016</td>
</tr>
<tr>
<td>4</td>
<td>RPMI + 5% dialyzed FBS + G418 1 g/L</td>
<td>500</td>
<td>1</td>
<td>22</td>
<td>Not applicable</td>
<td>1/1921</td>
</tr>
<tr>
<td>T+</td>
<td>RPMI + 5% dialyzed FBS + G418 1 g/L</td>
<td>100</td>
<td>1</td>
<td>10</td>
<td>Yes</td>
<td>1/910</td>
</tr>
<tr>
<td>T+</td>
<td>RPMI + 5% dialyzed FBS + G418 1 g/L</td>
<td>500</td>
<td>1</td>
<td>34</td>
<td>Yes</td>
<td>1/1144</td>
</tr>
</tbody>
</table>

3.2 Establishment and freezing of pools
The first pool of each cuvette is achieved at J+16 and the second one at J+19. These pools are titrated by ELISA (table 5).

Table 5: Establishment and titration of pools

<table>
<thead>
<tr>
<th>Date</th>
<th>Cuvette</th>
<th>Pool</th>
<th>Number of cells (x10^4)</th>
<th>Viability (%)</th>
<th>Titer (μg/mL)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12/12/08</td>
<td>1</td>
<td>1</td>
<td>0.6</td>
<td>71</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>0.54</td>
<td>84</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>0.5</td>
<td>86</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1</td>
<td>0.56</td>
<td>80</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>12/15/08</td>
<td>1</td>
<td>2</td>
<td>0.38</td>
<td>59</td>
<td>4.9</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>0.42</td>
<td>64</td>
<td>5.6</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>0.48</td>
<td>63</td>
<td>6.3</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>0.50</td>
<td>62</td>
<td>6</td>
<td>34</td>
</tr>
</tbody>
</table>

Pool No. 2 of each cuvette was amplified in RPMI medium, 5% dialyzed FBS, 0.5 g/L geneticine, 25 mM MTX for conservation. Two CryoTubes were done for each pool. Data about freezing are shown in table 6. A PCR-based Mycoplasma detection test was conducted on each sample on the day of freezing. Cells are free of Mycoplasma contamination.

Table 6: Conservation of pools

<table>
<thead>
<tr>
<th>Pool</th>
<th>Viability (%)</th>
<th>Number of cells (x10^6)</th>
<th>Number of CryoTubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuvette 1</td>
<td>85</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Cuvette 2</td>
<td>90</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Cuvette 3</td>
<td>93</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Cuvette 4</td>
<td>93</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

3.3 Production in roller culture

Quantities to be produced for each cuvette are shown in table 7.

Table 7: Quantities to be produced for each antibody

<table>
<thead>
<tr>
<th>Cuvette No.</th>
<th>Human IgG titration of transfected pools (μg/mL) in EMS</th>
<th>Ab quantity (mg) required for experiments</th>
<th>Ab quantity to be produced (mg)</th>
<th>Number of roller bottles (900 mL each)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuvette 1</td>
<td>4 3</td>
<td>10</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>Cuvette 2</td>
<td>6 4</td>
<td>120</td>
<td>140</td>
<td>24</td>
</tr>
</tbody>
</table>
The roller cultures were carried out in EMS medium, 5% FBS depleted of bovine Igs, 0.5 g/L geneticine.

The supernatants were titrated by ELISA at the end of the roller culture production (table 9).

Table 9: Titration of the supernatants of the roller cultures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/mL)</th>
<th>Produced quantity (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuvette 1</td>
<td>3.4</td>
<td>12.2</td>
</tr>
<tr>
<td>Cuvette 2</td>
<td>9.8</td>
<td>211.7</td>
</tr>
<tr>
<td>Cuvette 3</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Cuvette 4</td>
<td>6.1</td>
<td>16.5</td>
</tr>
</tbody>
</table>

The supernatants were concentrated by tangential ultrafiltration on Proflux (Millipore), filtered through a 0.22 µm filter. The recovered volumes after concentration are shown in table 10.

Table 10: Production volumes of the roller cultures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Produced volume (mL)</th>
<th>Concentrated volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuvette 1</td>
<td>3,600</td>
<td>619</td>
</tr>
<tr>
<td>Cuvette 2</td>
<td>21,600</td>
<td>1,764</td>
</tr>
<tr>
<td>Cuvette 3</td>
<td>9,000</td>
<td>621</td>
</tr>
<tr>
<td>Cuvette 4</td>
<td>2,700</td>
<td>621</td>
</tr>
</tbody>
</table>

Example 2 anti MUC-I production

The anti-MUC1 antibodies, which are contained in supernatants of the YB2/0 cell line production, were purified by gel affinity chromatography on Sepharose-protein A and then characterized.

The purification yields of the R764-hHMFG1 and R764-D11 antibodies, on a column that contains 5 mL of gel, are respectively of 74 and 73%.

The purification yields of the two other antibodies R764-B7 and R764-G2, on a column that contains 1 mL of gel, are respectively of 31 and 75%. 
MATERIALS AND METHODS

1 MATERIAL

2.1.1 Supernatants of production
- R764-722 08/017 cuvette 3; vector huHMFGl; volume: 3 liters
- R746-722 08/017 cuvette 2; vector HT1 86-D1; volume: 22 liters
- R764-722 08/017 cuvette 1; vector HT186-B7; volume: 9 liters
- R764-722 08/017 cuvette 4; vector HT186-G2; volume: 3 liters

Production medium: EMS + 5% FBS depleted of bovine IgGs + 1 g/L G418.

2.1.2 Chromatography columns
- HiTrap™ rProtein A FF 5 ml (GE Healthcare, 17-5080-01, Lot No. 10010319)
- HiTrap™ rProtein A FF 1 ml (GE Healthcare, 17-5079-01, Lot No. 10008107)

2 METHODS

2.1 IgG1 anti-MUC1 purification by affinity chromatography

After equilibration by addition of 10 column volumes of buffer A1 (Tris 25 mM, NaCl 25 mM, EDTA 5 mM, pH 7.10), the supernatant of culture is injected onto the Sepharose-protein A column. Afterwards the said column is washed by 15 volumes of buffer A1 and the retained antibodies in the Sepharose-rprotein A gel are eluted by injection of the buffer B1 (Sodium citrate 25 mM, pH 3.6). The eluted fractions from the 1 and 5 mL HiTrap columns are respectively of 1 and 3 mL, and are collected in tubes containing 1/10th of the volume of the Tris 2M pH 7.5 fraction.

After elution, the Sepharose-rprotein A columns are regenerated by injection of a 0.1M phosphoric acid solution during 3 minutes and then re-equilibrated with buffer A1.

For the 5 mL HiTrap column, flow rates of equilibration, injection and washing are of 5 mL/min and the elution flow rate is of 2.5 mL/min. For the 1 mL HiTrap column, the flow rate is of 0.5 mL/min except for the last 10 volumes of the washing step, for which the said flow rate of equilibration is increased to 1 mL/min.

The eluted fractions containing the antibody are pooled and dialyzed at 4°C against PBS (Sigma, P-4417) during one night. Then the eluate is filtered through a syringe filter, which has a porosity of 0.2 run.

2.2 Characterization methods
- Titration of Human IgGs: FastELYSA (RD biotech kit)
- O.D. (280 nm): the M.O. 403-041 1/2
- SDS-PAGE electrophoresis: the M.O. 613-202/1
- SDS-CGE: IGG purity kit according to the protocol given by the manufacturer
- Molecular weight distribution by gel filtration: the M.O. 643-0205
- Fucose titration: the M.O. 613-331/1 and 613-207/1. Fucose titration by ELISA-AAL

RESULTS AND COMMENTS

1 PURIFICATION OF THE ANTIBODIES

The supernatants of production were concentrated about 15 times by tangential ultrafiltration before purification. The rates of antibodies contained in the concentrated supernatants of production and in the dialyzed and filtered elutions were titrated by the FastELYSA technique in the same test, in order to evaluate the purification yields.

The IgGs contained in the supernatants of production of the R764-hHMFG1 and R764-D11 clones were purified by chromatography on a 5 mL column, while the IgGs from the R764-B7 and R764-G2 clones were purified on a 1 mL column. The yields of purification of the antibodies are about 74%, except for the R764-B7 antibody which has a yield of purification of 31% (Table I). The value of the yield of purification for the R764-B7 clone can be explained by the fact that, after elution and dialysis, the solution was very cloudy which required a centrifugal step before filtration. After this filtration step, the solution stored in a tube at 4°C got cloudy again.

Table I: Anti-MUC1 antibodies yields of purification

<table>
<thead>
<tr>
<th>Purification No.</th>
<th>Clone name</th>
<th>Vol. (mL)</th>
<th>IgG (mg)</th>
<th>Vol. (mL)</th>
<th>IgG (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>632-09/143</td>
<td>R764-hHMFG1</td>
<td>597</td>
<td>86.5</td>
<td>24.8</td>
<td>63.6</td>
<td>74</td>
</tr>
<tr>
<td>632-09/144</td>
<td>R764-D11</td>
<td>1,700</td>
<td>204</td>
<td>65</td>
<td>149</td>
<td>73</td>
</tr>
<tr>
<td>632-09/145</td>
<td>R764-B7</td>
<td>601</td>
<td>12</td>
<td>5</td>
<td>3.8</td>
<td>31</td>
</tr>
<tr>
<td>632-09/146</td>
<td>R764-G2</td>
<td>605</td>
<td>16</td>
<td>5.6</td>
<td>12</td>
<td>75</td>
</tr>
</tbody>
</table>
The preparations of antibodies to be injected into the animal for in vivo tests were titrated for endotoxins by the microbiology control laboratory. The quantities of endotoxins existing in the preparations of antibodies R764-hHMFGl and R764-D11 are respectively of 5 IU/mL and < 1.5 IU/mL.

3 PURIFIED ANTI-MUC1 ANTIBODIES CHARACTERIZATION

3.1 Electrophoretic control

The anti-MUC1 antibodies were electrophoresed through a polyacrylamide gel in order to determine on the one hand, their purity and on the other hand, their apparent molecular weight (MW\text{app}).

Under non-reducing conditions, the electrophoretic profile of the anti-MUC1 IgGs reveals a band that is in the majority, equal to 156 kDa and corresponding to the entire IgG (2H2L). The R764-D11 and R764-G2 antibodies (lanes No. 2 and 4) also show 3 minor bands of MW\text{app} values: 143, 114 and 80 kDa. The electrophoretic profile of the R764-B7 antibody (lane 3) is made up of 6 bands in addition to the band that is in the majority, and equal to 156 kDa (Fig. 8).

Under reducing conditions, the electrophoretic profile of the antibodies reveals 2 major bands, corresponding to the heavy chain (HC) and to the light chain (LC). The R764-B7 antibody shows in addition a double band of low intensity and of MW\text{app} equal to 36 and 37 kDa.

The MW\text{app} of the heavy and light chains of the R764-hHMFGl antibody differ significantly from those of the other anti-MUC1 antibodies: 56 vs. 54 for the heavy chain, and 28 vs. 29 for the light chain. The R764-hHMFGl antibody is an IgGl, kappa, while the 3 other antibodies are of isotype IgGl, lambda. In addition, the R764-hHMFGl antibody has a very different primary structure compared to the 3 other antibodies, which differ only by 5 to 6 amino acids per chain.

3.2 SDS-PAGE

In order to establish the relative proportions of the different antibody forms existing in the preparations, an analysis by capillary electrophoresis is done under non-reducing and reducing conditions.

In non-reducing conditions, the anti-MUC1 antibodies show percentages of entire IgGs (2H2L) that are between 62.5 and 79.2%. The quantity of the form 2HIL is smaller in
the R764-hHMFG1 antibody preparation than in other preparations where it represents about 15 to 18% of the total forms.
Table II: Percentages of the different forms of the IgG molecule existing in the purified preparations of antibodies

<table>
<thead>
<tr>
<th>Sample</th>
<th>Antibody</th>
<th>IgG</th>
<th>2H1L</th>
<th>HH</th>
<th>HC</th>
<th>LC</th>
<th>UD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>632-09/143</td>
<td>R764-hHMFG1</td>
<td>79.2</td>
<td>3.2</td>
<td>-</td>
<td>0.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>632-09/144</td>
<td>R764-D11</td>
<td>70.3</td>
<td>16.2</td>
<td>4.3</td>
<td>-</td>
<td>2.6</td>
<td>7.6</td>
</tr>
<tr>
<td>632-09/145</td>
<td>R764-B7</td>
<td>62.5</td>
<td>14.7</td>
<td>4.3</td>
<td>8.3</td>
<td>6.3</td>
<td>7.9</td>
</tr>
<tr>
<td>632-09/146</td>
<td>R764-G2</td>
<td>69</td>
<td>18</td>
<td>4.5</td>
<td>0.5</td>
<td>2.1</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*UD*: undetermined peak

After action of a reducing agent, the heavy chains (HC) and light chains (LC) of the antibodies are separated by capillary electrophoresis. This method allows detecting and establishing the percentage of non-glycosylated heavy chain (NG-HC).

The NG-HC quantity existing in the antibodies produced by the YB2/0 cell line is generally < 1%, like in the 3 other anti-MUC1 antibodies: R764-D11, R764-B7, and R764-G2. On the other hand, the R764-hHMFG1 antibody contains a rate of non-glycosylated heavy chain that is about 5.4%, which is 10 times superior to the rate of the other anti-MUC1 antibodies.

Table III: Percentages of non-glycosylated chains of IgGs existing in the preparations of antibodies after reduction

<table>
<thead>
<tr>
<th>Antibody</th>
<th>LC</th>
<th>HC</th>
<th>NG-HC*</th>
<th>UD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>R764-hHMFG1</td>
<td>40.6</td>
<td>54</td>
<td>5.4</td>
<td>-</td>
</tr>
<tr>
<td>R764-D11</td>
<td>33.8</td>
<td>62.3</td>
<td>0.4</td>
<td>3.5</td>
</tr>
<tr>
<td>R764-B7</td>
<td>40.4</td>
<td>57.5</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>R764-G2</td>
<td>35.7</td>
<td>63.9</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

*NG-HC*: non-glycosylated heavy chain; *UD*: undetermined peak

3.3 Molecular weight determination

The chromatograms obtained after injection of the purified anti-MUC1 IgG preparations on the gel filtration column show a peak that is in the majority, with a retention time of 32.5 min (± 0.5 min). This retention time corresponds to the monomer of the human IgGl. Minor peaks with retention times of 27.3 (± 0.3) and 21.8 (± 0.4) minutes also exist; these peaks correspond to dimers and multimers of human IgGIs respectively.

Table IV: Percentages of isomers of anti-MUC1 antibodies
The results of Table IV show that the 4 anti-MUC1 antibodies have a rate of monomer that is superior to 95%.

3.4 Fucose titration

The titers of the α(l-6) linked fucose are determined by the lectin ELISA method. The results shown in Table V indicate that the 3 antibodies R764-D1, R764-B7 and R764-G2 have similar fucose rates, close to 30%. On the other side, the R764-hHMFG1 control antibody has a fucosylation rate that is 2.5 times superior.

Table V: α(l-6) linked Fucose rates determined by the lectin ELISA method

<table>
<thead>
<tr>
<th>Antibody</th>
<th>779-09/038</th>
<th>779-09/039</th>
<th>779-09/043</th>
<th>779-09/044</th>
<th>Average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>R764-hHMFG1</td>
<td>80.7</td>
<td>-</td>
<td>-</td>
<td>82</td>
<td>81 (± 0.9)</td>
</tr>
<tr>
<td>R764-D11</td>
<td>-</td>
<td>-</td>
<td>28.2</td>
<td>25.2</td>
<td>27 (± 2.1)</td>
</tr>
<tr>
<td>R764-B7</td>
<td>33</td>
<td>27.0</td>
<td>29.7</td>
<td>28.3</td>
<td>29 (± 2.6)</td>
</tr>
<tr>
<td>R764-G2</td>
<td>-</td>
<td>26.0</td>
<td>32.1</td>
<td>26.8</td>
<td>28 (± 3.3)</td>
</tr>
</tbody>
</table>

3.3.5 Antigenic recognition by ELISA test

This analysis was done using the ELISA technique wherein the 32-mer MUC1 peptide is immobilized in the wells of a microtitre plate, and wherein the anti-MUC1 antibodies are added later in different concentrations. The fixed antibodies are revealed and the O.D. values
are reported to a graph on the Y-axis, while the antibody quantities are reported on the X-axis (Fig. 9).

CONCLUSION

The 4 anti-MUC1 antibodies were purified by gel affinity chromatography on Sepharose-protein A. Because of the different quantities of antibodies to be purified, it was necessary to adapt the size of each affinity column. The R764-hHMFG1 and R764-D11 antibodies were purified on a 5 mL gel column, and their yields of purification were of 74 and 73% respectively. The 2 other antibodies, R764-B7 and R764-G2 were purified on a 1 mL gel column, and their yields of purification are 33 and 75% respectively. The poor yield of purification of the R764-B7 antibody can be explained by the existence of a precipitate after the dialysis step, which required a centrifugal step before filtration.

The analysis of the antibodies by CGE or PAGE electrophoresis, under non-reducing conditions, show that the R764-hHMFG1 which is of isotype IgG1, kappa is less sensible to light chain dissociation than the 3 other antibodies that are of isotype IgG1, lambda. However, if one refers to the chromatography results of molecular weight distribution, it appears that the 4 antibodies have a similar distribution of molecular weights, except the R764-B7 antibody, which has a rate of multimers superior to the others (3.56% vs. 0-0.9%).

The analysis of the antibodies by CGE or PAGE technique, under reducing conditions, show that the R764-hHMFG1 has a rate of non-glycosylated heavy chain that is significatively superior (5.4%) to the 3 other antibodies (<1%). In addition, this antibody show a rate of $\alpha$(I-6) linked fucose, which was determined by the lectin ELISA technique, that is very high (81%) in comparison to the 3 other antibodies, which have a rate of fucose that is about 30%.

The antigenic recognition done with the ELISA technique show that the R764-D11 and R764-B7 antibodies bind 10 times more strongly to the 32-mer MUC1 peptide than the 2 other antibodies, R764-G2 and R764-hHMFG1.

To conclude, we note that the anti-MUC1 antibodies coming from the maturation step of the scFv IIIB6, though they have quite similar primary structures, behave differently with respect to their antigenic recognition (R764-G2 differs from the 2 others) and the stability of the IgG molecule (R764-B7 seems less "stable" than the 2 others). The R764-hHMFG1 control antibody whose primary structure differs from the antibodies coming from the scFv
IIB6, shows an "atypical" glycosylation with respect to the other antibodies produced by the YB2/0 cell line.

**Example 3 ELISA with anti-MUC1 IgG on MUC1 peptide antigen**

- Dilution series of anti-MUC1 IgG (D1, 1, B7, G2)
- 3x Nunc Maxisorp plates coated with 50 ng/well MUC1 peptide antigen (32mer cys, sequence: ADPTRP APGSTAPP AHGVSAPDTRP APGSTAC )
- Control plate was coated with 100 ng/well BSA
- Blocking solution: 2% skim milk powder in PBS-T (0.1 % Tween), 1 h, RT
- IgG samples were diluted to a final concentration of 100 µg/ml with blocking solution
- samples were sequentially diluted 1 : 3.16
- detection: goat anti-human IgG (Fc spec) HRP conjugate (Sigma, 1:39000), 1 h, RT
- Development: TMB substrate, reaction stopped with sulforic acid after 10 minutes.
- A450 - A620 measured in a Tecan Sunrise Reader

- Calculations performed:
  - Mean value and standard deviation of three plates (MUC1) calculated
  - BSA value (negative control) substracted from mean value
  - Plotting:

Mean values were plotted against antibody concentration, SD is given by vertical error bars

Results are presented in figure 10

**Example 4: FACS analysis of anti-MUC1 antibodies on MUC1 positive cell line T47D.**

**Cell lines**

T47D: ATCC No. HTB-133, MUC-I positives

**Culture conditions**

The cells were grown in 10 cm polystrol dishes at 37 °C, 5 % CO₂ in DMEM medium (4.5 g/L glucose) supplemented with 8 % FCS and 1 % Penicillin/Streptomycin stock solution (10Ox)

**Cell counting**

Cells were:
- washed with PBS (10 mL)
- solubilized by adding 1 mL trypsin/EDTA to each 10 cm dish
- Incubating for 5-10 min at RT
- resuspended by carefully pipetting up and down
- cells of all four plates were transferred to 5 mL medium
- dilution of cells with brome phenol blue solution
- 10 µL of the brome phenol blue—cell mixture were analyzed in an Neubauer counting chamber, 4 big squares (factor 1x 10^4 each) were counted

<table>
<thead>
<tr>
<th>Cell line</th>
<th>dilution</th>
<th>1st big square</th>
<th>2nd big square</th>
<th>3rd big square</th>
<th>4th big square</th>
<th>total</th>
<th>conc. cells/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47D</td>
<td>1:2</td>
<td>71</td>
<td>70</td>
<td>69</td>
<td>67</td>
<td>138</td>
<td>1.38 x 10^6</td>
</tr>
</tbody>
</table>

For each cell staining 1.4 x 10^5 cells should be used. Therefore the following volume of the cell suspension is needed:

T47D: 101 5 µL (each)

The following antibodies were used for specific MUC1 staining:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration (µg/mL)</th>
<th>Sample Amount (µL)</th>
<th>Buffer Amount (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IgG1 (kappa) (Sigma, I5154) (iso)</td>
<td>830</td>
<td>17.6</td>
<td>128.6</td>
</tr>
<tr>
<td>hHMFG1 IgG</td>
<td>2563</td>
<td>5.7</td>
<td>140.5</td>
</tr>
<tr>
<td>D11 IgG</td>
<td>2294</td>
<td>6.4</td>
<td>139.8</td>
</tr>
<tr>
<td>B7 IgG</td>
<td>755</td>
<td>194</td>
<td>126.8</td>
</tr>
<tr>
<td>G2 IgG</td>
<td>2152</td>
<td>68</td>
<td>139.4</td>
</tr>
</tbody>
</table>

From these antibody solutions further dilution by a factor of 3.162 were made (in a PP microtiter plate), so following antibody concentrations were used for FACS (µg/mL): 100, 31.62 10, 3.16; 1, 0.316; 0.1, 0.0316, 0.01, 0.003162

bound anti-MUC1 antibodies were detected by.
goat anti-human IgG (H+L) Alexa488 conj. 1:200 = 10 µg/mL final (Invitrogen resp. Molecular probes Cat.No. A-1 1013) in 100 µL FACS buffer in total: 38.5 µL (stock: 2 mg/mL) were given to 7661.5 µL FACS buffer

5 Cell staining
The above given volumes of cell suspension were given into 5 mL PP tubes (Greiner)
- add 3 mL FACS buffer (PBS + 2 % FCS + 2 mM EDTA, ice cold) each
- centrifuge cells: 300 xg, 4 min, 4 °C
- incubate 100 µL anti-MUC1 Abs (30 µg/mL), 1h on ice
- centrifuge cells: 300 xg, 4 min, 4 °C
- wash with 3 mL FACS buffer (ice cold)
- centrifuge cells: 300 xg, 4 min, 4 °C
- wash with 3 mL FACS buffer (ice cold)
- incubate 100 µL detection antibody (goat anti-human IgG (H+L) Alexa488 conj.), 1 h on ice
- centrifuge cells: 300 xg, 4 min, 4 °C
- wash with 3 mL FACS buffer (ice cold)
- centrifuge cells: 300 xg, 4 min, 4 °C
- wash with 3 mL FACS buffer (ice cold)
- resuspend cells in 500 µL FACS buffer - store on ice until measurement

Measurement
The measurement was performed on a Beckman Coulter Cytomics FC500. Gates were set by analyzing a mock-control of each cell line w/o any antibody incubation. Specific protocol files (pro) were saved.
10000 cells were analyzed per run.

<table>
<thead>
<tr>
<th>Concentration µg/ml</th>
<th>D11 IgG MFI</th>
<th>B7 IgG MFI</th>
<th>G2 IgG MFI</th>
<th>hHMFG1 IgG MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.0000</td>
<td>17.4</td>
<td>12.6</td>
<td>6.15</td>
<td>3.58</td>
</tr>
<tr>
<td>31.6228</td>
<td>13.2</td>
<td>9.16</td>
<td>3.68</td>
<td>2.45</td>
</tr>
<tr>
<td>10.0000</td>
<td>11.1</td>
<td>6.56</td>
<td>1.99</td>
<td>1.23</td>
</tr>
<tr>
<td>3.1623</td>
<td>7.92</td>
<td>4.96</td>
<td>0.802</td>
<td>1.16</td>
</tr>
<tr>
<td>1.0000</td>
<td>4.47</td>
<td>2.92</td>
<td>0.605</td>
<td>1.125</td>
</tr>
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Results are presented in Figure 11 to Figure 21.

Example 5 ADCC using NK cells as effector cells

ADCC (Antibody-Dependent Cellular Cytotoxicity) with or without pre sensitisation consists to contact target cells, effector cells and antibodies (specific of the target cells)
Cytotoxic activity mediated by the used antibodies is revealed by colorimetry: lactate dehydrogenase (LDH) dosage which is released by lysed cells or by measuring the release of another constituent incorporated into target cell (AM calcein)

Protocol
1 - reagents
HBSS IX for instance Invitrogen ref: 14170-088
Ficoll-Paque™ PLUS for instance GE Healthcare ref: 17-1440-03
DPBS for instance Invitrogen ref : 14190-094
NH4Cl 157 mM, EDTA 0.099 mM, KHCO3 9.99
EMS - 5% SVF
NK Cell Isolation Kit - Miltenyi Biotec ref : 130-092-657
AutoMACS™ Running Buffer - Miltenyi Biotec ref : 130-091-221
AutoMACS™ Pro Washing Solution - Miltenyi Biotec ref : 130-092-987
IgGl-PE for instance Beckman Coulter ref : A07796
CD3-PE for instance Beckman Coulter ref : A07747
CD14-PE for instance Beckman Coulter ref : A07764
CD16-PE for instance Beckman Coulter ref : A07766
CD19-PE for instance Beckman Coulter ref : A07769
CD56-PE for instance Beckman Coulter re : A07788
AM calcein 1 mg/ml in DMSO for instance Invitrogen ref : C3099
EMS - 5% SVF - 4 mM sulfinpyrazone
EMS - 5% SVF - 2% Triton X100
Cytotoxicity Detection Kit for instance ROCHE ref : 11644 793 001
Blood sample extraction

Blood is diluted twice with HBSS IX.
Diluted blood is aliquoted in 50 mL tubes containing:
- 15 ml of Ficoll-Paque™ PLUS at room temperature
- 30 ml of diluted blood,

Centrifugation 530 g, 20 minutes RT

• Extraction of mononuclear cells
  with a pipette, mononuclear cells are gently collected and transferred to a 50mL tube containing HBSS IX
  MNC are washed by centrifugation at 480 g for 10 minutes at RT

Discard supernatant and pellets are pooled in HBSS IX.

• Platelet elimination
  Cells are centrifuged at 190 g, 15 minutes RT

• Red cells elimination
  Supernatant is discarded and pellets are resuspended in 50 ml of NH4Cl, and left at RT for 5 min under agitation.
  Cells are centrifuged at 480 g 5 min RT.
  Cells are washed once with HBSS IX by centrifugation at 480 g 5 minutes RT
  Discard supernatant.

• Counting

Cells are counted in a Malassez cell.

• NK purification by negative selection
  for 100x10^6 MNC
  D 0,4 ml of cold AutoMACS Running Buffer is added and 0,1 ml of the NK Cell Biotin Antibody Cocktail is added
  D Leave at 4°C for 10 min
  D 0,3 ml cold AutoMACS Running Buffer + 0,2 ml of NK Cell MicroBead Cocktail are added
  D Leave at 4°C for 15 min
D Add 1 to 2 ml of cold AutoMACS Running Buffer.
D Centrifugation 300 g 10 minutes, + 4°C
D Discard supernatant.
D Pellet is resuspended with 500 µl of cold AutoMACS Running Buffer for 100x10^6 cells.
D Filtration on 30 µm filter previously incubated with AutoMACS Running Buffer.
D Place on AutoMACS Pro and run the ad hoc program:

- **Facs NK phenotyping**
  D Cell labelling (1 well by dye)
  put 50 µl of NK cell suspension + 5 µl of antibody coupled with PE (IgGl; CD3; CD14; CD 16; CD 19; CD56) in a 96-well plate.

  Leave on ice for 10 min
  cells are washed twice by centrifugation 330g 10 min
  cells are fixed in 300 µl DPBS containing 1% PFA.

- **Preparation of target cells**
  - Wash by addition of DPBS and centrifuge at 480 g, at room temperature during 5 minutes
  - Eliminate the supernatant and put the centrifugation pellet into EMS + 5% FBS medium in order to obtain:
    - in case of a lactate dehydrogenase (LDH) revelation test: 6x10^5 cells/mL
    - in case of a Calcein-AM revelation test: 3x10^6 cells/mL
  - To 1 mL of cell suspension containing between 1 and 3x10^6 cells/mL in EMS + 5% FBS medium, add 25 µL of Calcein-AM at 1 mg/mL in DMSO solution.
  - Incubate at 37°C - 7% CO₂ during 20 minutes
  - Fill the tube with the EMS + 5% FBS medium.
  - Centrifuge at 480 g and at room temperature during 5 minutes
  - Gently resuspend the centrifugation pellet in EMS + 5% FBS medium and fill the tube with this medium
  - Centrifuge at 480 g and at room temperature during 5 minutes
Discard the supernatant and gently resuspend the centrifugation pellet in order to obtain a solution which has a concentration of $3 \times 10^5$ cells/mL in EMS + 5% FBS medium + 4 mM sulphinpyrazone, preheated to 37°C.

5. **Preparation of the antibodies**

Dilute the antibodies that are in the EMS + 5% FBS medium to a concentration of 20 µg/mL. Then dilute from one in ten to one in ten with a new pipette tip for each dilution.

**ADCC test**

1. Put 50 µL/well for each antibody to be tested
2. Add 50 µL/well of the target cell suspension
3. Add 50 µL/well of the effector cell suspension
4. Add EMS + 5% FBS medium to obtain a final volume of 200 µL

One obtain for each well a cell ratio $R$, with:

$$R = \text{effector cells/target cells} = \frac{X}{Y}$$

The ideal ratio is $R = 15/1$. According to the number of antibodies to be tested and to the number of recovered NK cells, each condition is tested in duplicate or triplicate.

**Control wells (duplicate or triplicate of wells)**

1. **Standard reference range: 100%, 50%, 25% and 0% of lysis (duplicate or triplicate)**
   1. **100%**: 50 µL of target cells + 50 µL of EMS containing 5% of FBS + 100 µL of Triton X100 at 2%
   2. **50%**: 25 µL of target cells + 75 µL of EMS containing 5% of FBS + 100 µL of Triton X100 at 2%
   3. **25%**: 12.5 µL of target cells + 87.5 µL of EMS containing 5% of FBS + 100 µL of Triton X100 at 2%
   4. **0%**: 100 µL of EMS containing 5% of FBS + 100 µL of Triton X100 at 2%

**The control cell targets**

50 µL of target cells + 150 µL of EMS containing 5% of FBS

**The control effector cells**

50 µL of effectors cells + 150 µL of EMS containing 5% of FBS

**The control AICC**
50 µL of target cells + 50 µL of effectors cells + 100 µL EMS containing 5% of FBS

The control antibodies
50 µL of antibodies at the different target cell concentrations + 50 µL of target cells + 100 µL of EMS containing 5% of FBS

After the distribution of all constituents:
- Gently agitate
- Centrifuge at 125 g and at room temperature during 1 minute
- Incubate the plates at 37°C and 7% CO2 during 4 hours for a Calcein-AM revelation or during one night for a LDH revelation test.

A - Colorimetric method for the LDH titration

After incubation at 37°C and 7% CO2 during one night:
- Centrifuge the plates at 125 g at room temperature during 1 minute
- Gently sample 130 µL of supernatant of every well and transfer them to a 96-well round-bottomed plate with a multichannel pipette
- Centrifuge these plates again at 125 g and at room temperature during 1 minute
- Gently sample 50 µL of supernatant of every well and transfer them to a 96-well flat-bottomed plate with a multichannel pipette
- In these 50 µL of supernatants, gently add (to avoid the formation of air bubbles) 50 µL of the revealing solution of the cytotoxicity detection kit (dilute the first reagent to 1:45 in the second reagent of the kit).
- Incubate at room temperature in the dark for 30 minutes
- Add 100 µL/well of HCl IN in order to stop the enzymatic reaction
- If necessary, eliminate the air bubbles with a single use needle
- Gently agitate, and read the O.D. at 492 nm with help of the BIOLISE software

B - Calcein-AM method using a fluorimeter

After incubation at 37°C and 7% CO2 during 4 hours:
- Centrifuge the plates at 125 g at room temperature during 1 minute
- Gently sample 150 µL of supernatant of every well and transfer them to a 96-well round-bottomed plate with a multichannel pipette
- Centrifuge these plates again at 125 g and at room temperature during 1 minute.
- Sample 100 µL of supernatants and gently transfer them in a black 96-well flat-bottomed plate.
- At this stage, the plates can be stored in a refrigerator for a maximum of 24 hours.

- Put the plate in the fluorimeter: excitation at 485 nm and emission at 535 nm. Read with help of the BIOLISE software.

The results are shown in the figure 22.

10 Annex 1 — Preparation of solutions

A EMS - 5% FBS

95 mL EMS for instance Invitrogen Ref: 041-95181M
5 mL FBS for instance Invitrogen Ref: 011-90018M 5%
Can be stored in the refrigerator for 1 month

B EMS - 5% FBS - Triton X100 2%

5 µL EMS - 5% FBS (Solution A)
100 µL Triton X100 for instance Sigma Ref: 9002-93-1 2%
Can be stored in the refrigerator for 1 month

C NH₄Cl 157 mM, EDTA 0.099 mM, KHCO₃ 9.99 mM

See the M.O. No. 623/04635
Can be stored in the refrigerator for 3 years

D HCl 1 N

1,000 mL of demineralised water
91 mL of concentrated HCL 37%, for instance Merck Ref: 1.00314.1000 1 N
Can be stored at room temperature for 1 year

30 E NaOH 1 N

90 mL of demineralised water
10 mL of NaOH 30%, for instance Prolabo Ref: 28226293 1 N
Can be stored at room temperature for 1 year

F sulphinpyrazone 0.1 M
0.2420 g of sulphinpyrazone for instance Sigma Ref: S9509 0.1 M
Q.S. 6 ml NaOH 1 N (Solution E) 1 N
Can be stored in the refrigerator for 2 months

G EMS - 5% SVF - 4 mM sulphinpyrazone
48 ml EMS - 5% FBS (Solution A)
2 ml sulphinpyrazone 0.1 M (Solution F) 4 mM
150 µl of concentrated HCl 37%, for instance Merck: 1.00314.1000 pH 7.5
Filter on a 0.2 µm.
Can be stored in the refrigerator for 1 month
Remark: The sulphinpyrazone is a blocker of the calcium channels. It is used to avoid the spontaneous release of the Calcein-AM

H DPBS - 4% PFA
4 g of paraformaldehyde for instance Sigma P6148 4%
Q.S. 100 ml DPBS for instance Invitrogen Ref: 14190-094
Make aliquots of 1 mL
Can be stored in the freezer for 1 month

I DPBS - 1% PFA
1 ml of DPBS - 4% PFA (Solution H) 1%
3 ml of DPBS for instance Invitrogen Ref: 14190-094
Can be stored in the refrigerator for 1 month

Example 5 Determination of *in vitro* serum stabilites of anti-MUC1 IgG or anti-MUC1 scFv-Fc fusion proteins.

Used antibodies

- 632 09 143 R764 huHMFGl 785 µl 2563 µg/ml
- 632 09 144(2) R764 HTl 86-Dl 1 875 µl 2294 µg/ml
- D11 scFv-Fc (HT351) 0.62 mg/ml
Preparation

5 ml antibody solution (in PBS) were prepared each, cone. 20 µg/ml

hHMFGl : 39 µl + 4961 µl

D1 HgG : 43,6 µl + 4956,4 µl
D11 scFv-Fc : 161,3 µl + 4839 µl

Each dilution was prepared 3x to have material for triplicates.

100 µl of these solutions were mixed with either 100 µl PBS (incl. 1 % Pen/Strep, PAA laboratories) or human serum (off the clot, PAA laboratories Cat.No. C11-020, Lot. C02007-0863 suppl. with 1 % Pen/Strep and 0,1 % Sodium azide) and human serum inactivated for 30 min at 56 °C.

The mixtures were put into tubes and freeze-dried at -80 °C.
On specific time point aliquots were incubated at 37 °C.

20.5. 127.5. 130.5. | 3.6. | 5.6. | 9.6. | 12.6. | 16.6. | 17.6.

ELISA

MUC1 peptide (32 mer cys) was coated with 50 ng per well in microtiter plates. Wells were blocked with 2 % skim-milk powder in PBS-T (0,1 % Tween20). (BSA 100 ng/well as negative control).

50 µl of each aliquot were mixed with 50 µl blocking solution and incubated for 1 h at RT.
A goat anti-human IgG (Fc spec. HRP conj.) was used as detection antibody.

TMB development was done for 9 min.

Results for D11 IgG, D11 scFv FC and D11 hHMFGl IgG are presented respectively in figures 23, 24 and 25.
Example 6: Antibody internalisation

Cells: MCF-7 cell lines expressing MUC-I

Antibody is added to target cell

Antibodies and cells are incubated at 37°C for 1 hour, or 4°C for the control.

Cells are washed for removing free antibodies.

Secondary antibody is added for 1 hour at 4°C

Cells are washed for removing free antibodies.

Fluorescence is measured by using FACS.

Results are shown in figure 26.

The results show that about 70% of the antibodies (DI 1 or control antibody FG1) are internalized after 1 hour of incubation at 37°C on MCF7 cells.
1. Binding peptide having an affinity for the epitope formed by amino acid sequence RPAP of a peptide, characterized by the binding peptide comprising a heavy chain variable region amino acid sequence

\[ \text{FRI - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4} \]

wherein CDR1 is selected from SEQ ID NO: 1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO: 5 and SEQ ID NO: 6,

CDR2 is selected from SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12, and

CDR3 is selected from SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18,

and in association with the heavy chain amino acid sequence, a light chain variable amino acid sequence

\[ \text{FRI - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4} \]

wherein CDR1 is selected from SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24,

CDR2 is selected from SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30, and

CDR3 is selected from SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35 and SEQ ID NO: 36.

2. Binding peptide according to claim 1, wherein the heavy chain

\[ \text{FRI is selected from SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41 and SEQ ID NO: 42,} \]

FR2 is selected from SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47 and SEQ ID NO: 48,

FR3 is selected from SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53 and SEQ ID NO: 54,

FR4 is selected from SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59 and SEQ ID NO: 60,
and light chain
FR1 is selected from SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO:
64, SEQ ID NO: 65 and SEQ ID NO: 66,
FR2 is selected from SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO:
70, SEQ ID NO: 71 and SEQ ID NO: 72,
FR3 is selected from SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO:
76, SEQ ID NO: 77 and SEQ ID NO: 78,
FR4 is selected from SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO:
82, SEQ ID NO: 83 and SEQ ID NO: 84.

3. Binding peptide according to one of the preceding claims wherein the heavy chain
variable amino acid sequence is selected from the group consisting of SEQ ID NO: 85,
SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89 and SEQ ID NO: 90,
and the light chain variable amino acid sequence is independently selected from the
group consisting of SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO:
94, SEQ ID NO: 95 and SEQ ID NO: 96.

4. Binding peptide according to one of the preceding claims, wherein each variable
amino acid sequence is directly linked to at least one C-terminal constant regions.

5. Binding peptide: according to claim 4, wherein the constant region is selected from
SEQ ID NO: 97, SEQ ID NO: 98. SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO:
101. SEQ ID NO: 102. SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ
ID NO: 112, SEQ ID NO: 113 and SEQ ID NO: 114.

6. Binding peptide according to one of claims 4 and 5, wherein the heavy chain variable
amino acid sequence is directly linked to a C-terminal constant region selected from
SEQ ID NO: 97 to SEQ ID NO: 102, and
wherein the light chain variable amino acid sequence is directly linked to a C-terminal
constant region selected from constant region selected from SEQ ID NO: 109 to SEQ
ID NO: 114.
7. Binding peptide according to one of the preceding claims, which binding peptide is a single chain peptide, preferably said single chain peptide being selected from SEQ ID NO: 122 to SEQ ID NO: 124.

8. Binding peptide according to one of claims 1 to 6, wherein the light chain variable amino acid sequence is comprised on a first peptide and the heavy chain variable amino acid sequence is comprised on a second peptide, preferably said first peptide being selected from SEQ ID NO: 125 to SEQ ID NO: 130, and said second peptide being selected from SEQ ID NO: 131 to SEQ ID NO: 136.

9. Binding peptide according to one of the preceding claims, wherein at least one of the heavy chain amino acid sequence and the light chain amino acid sequence is linked to an effector selected from a detectable label selected from a dye, a radionuclide, a toxin, a cytotoxic enzyme.

10. Pharmaceutical composition for use in medical treatment of adenocarcinoma, comprising a binding peptide according to one of the preceding claims.

11. Process for analysis comprising the steps of contacting a sample suspected of containing a component comprising MUC1 epitope with a binding peptide according to one of claims 1 to 9, and detecting binding peptide bound to a component of the sample.

12. Process according to claim 11, wherein the sample is a biopsy of human origin.

13. Process for production of a peptide specifically binding to MUC1, characterized in that the peptide is a binding peptide according to one of claims 1 to 9.

14. Process according to claim 13, characterized in that the binding peptide is expressed in a cultivated cell containing a nucleic acid sequence encoding the binding peptide in an expression cassette.

15. Process according to one of claims 13 and 14, characterized in that the cultivated cell
is a mammalian cell, a fungal cell, a yeast cell or a bacterial cell.

16. Process according to one of the claims 12 to 15, characterized in that the coding sequence contains a sequence encoding a signal peptide for export of the binding peptide out of the cultivated cell's cytoplasm.
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N' IgG1 CH1 and N' IgG1 CL mean that only the first N-terminal amino acids of the CH1 or CL region respectively were present in the scFv. This is due to cloning and stabilization reasons.

Maybe it is more precise if it will be mentioned as “N-terminal amino acids of CH1 / CL region present in scFv”?
Fig. 4 A)

![Graph showing relative response over time for HT186-D11 Chip 1 with different concentrations.]

Fig. 4 B)

![Graph showing relative response over time for HT200-3A-C1 Chip 1 with different concentrations.]

Relative Response [RU]

0 100 200 300 400 500

0 200 400 600 800 1000 1200

t [s]

200 nM

100 nM

80 nM

60 nM

40 nM

20 nM

10 nM

5 nM

2 nM

250

200

150

100

50

0

200 nM

100 nM

80 nM

60 nM

40 nM

20 nM

10 nM

5 nM

2 nM

0 200 400 600 800 1000 1200

t [s]
Fig. 4 E)  

HT186-B7  
Chip 1  

Relative Response [RU]  

200 nM  
100 nM  
80 nM  
60 nM  
40 nM  
20 nM  
10 nM  
5 nM  
2 nM  

0  
200  
400  
600  
800  
1,000  
1,200  

0  
200  
400  
600  
800  
1,000  
1,200  

Fig. 4 F)  

HT186-G2  
Chip 1  

Relative Response [RU]  

200 nM  
100 nM  
80 nM  
60 nM  
40 nM  
20 nM  
10 nM  
5 nM  
2 nM  

0  
200  
400  
600  
800  
1,000  
1,200  

0  
200  
400  
600  
800  
1,000  
1,200  

[Graph showing data points and curves for HT186-B7 and HT186-G2 over time (t [s]) with relative response in RU for different concentrations.]
Fig. 6 G)

IIb6 scFv

- 5 µg mL\(^{-1}\)
- 1,6 µg mL\(^{-1}\)
- 0,5 µg mL\(^{-1}\)
- 0,16 µg mL\(^{-1}\)
- 0,05 µg mL\(^{-1}\)
- 0,016 µg mL\(^{-1}\)
- 0,005 µg mL\(^{-1}\)

\[ A_{450} - A_{620} \]

\[ t \,[d] \]

0  5  10  15  20  25  30
Figure 10

Figure 11
Overlay Plot 1

Figure 19

Events

FL1 Log

A
Figure 22

ADC91 09017b - NIH OVCar3 - Ratio E/C = 10/1
Figure 23

D11 IgG

Figure 24

D11 scFv Fc

Figure 25

hHMFG1 IgG
Figure 26
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/30

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

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

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

EPO-Internal, WPI Data, EMBASE, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X Further documents are listed in the continuation of Box C

X See patent family annex

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document relating to an oral disclosure of use exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance the claimed invention cannot be considered to involve an inventive step when the document is combined a with one or more other such documents such combination being obvious to a person skilled in the art

"A" document member of the same patent family

Date of the actual completion of the international search

11 November 2009

Date of mailing of the international search report

20/11/2009

Name and mailing address of the ISA/European Patent Office P B 5818 Patentlaan 2 NL - 2280 HV RIVM Tel (+31-70) 340-2040 Fax (+31-70) 340-3016

Authorized officer

Luyten, Kattie
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Form PCT/ISA/210 (patent family annex) (April 2005)