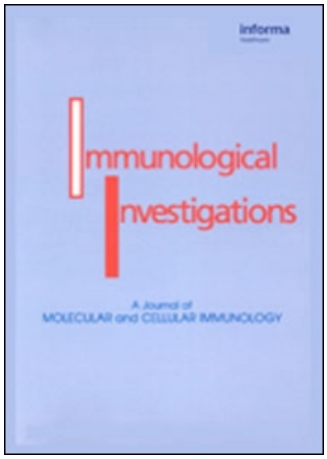

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The Production and Characterization of Novel Heavy-Chain Antibodies Against the Tandem Repeat Region of MUC1 Mucin

Fatemeh Rahbarizadeh,[1] Mohammad J. Rasaee,[1] Mehdi Forouzandeh,[2] Abdolamir Allameh,[1] Ramin Sarrami,[3] Habib Nasiry,[2] and Majid Sadeghizadeh[4]

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Camelidae are known to produce immunoglobulins (Igs) devoid of light chains and constant heavy-chain domains (CH_1). Antigen-specific fragments of these heavy-chain IgGs (VHH) are of great interest in biotechnology applications. This paper describes the first example of successfully raised heavy-chain antibodies in *Camelus dromedarius* (single-humped camel) and *Camelus bactrianus* (two-humped camel) against a MUC1 related peptide that is found to be an important epitope expressed in cancerous tissue. Camels were immunized against a synthetic peptide corresponding to the tandem repeat region of MUC1 mucin and cancerous tissue preparation obtained from patients suffering from breast carcinoma. Three IgG subclasses with different binding properties to protein A and G were purified by affinity chromatography. Both conventional and heavy-chain IgG antibodies were produced in response to MUC1-related peptide. The elicited antibodies could react specifically with the tandem repeat region of MUC1 mucin in an enzyme linked immunosorbant assay (ELISA). Anti-peptide antibodies were purified after passing antiserum over two affinity

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chromatography columns. Using ELISA, immunocytochemistry and Western blotting, the interaction of purified antibodies with different antigens was evaluated. The antibodies were observed to be selectively bound to antigens namely: MUC1 peptide (tandem repeat region), human milk fat globule membrane (HMFG), deglycosylated human milk fat globule membrane (D-HMFG), homogenized cancerous breast tissue and a native MUC1 purified from ascitic fluid. K_a values of specific polyclonal anti-peptide antibodies were estimated in C. dromedarius and C. bactrianus, as 7×10^{10} M^{-1} and 1.4×10^{10} M^{-1} respectively.

Keywords Heavy-chain antibody, MUC1, *Camelus bactrianus*, *Camelus dromedarius*.

INTRODUCTION

Hamers-Casterman et al. (1993) reported a novel class of two-heavy chain IgG antibodies, in addition to conventional four-chain IgG in Camelidae species, the only extant species of the Tylopoda suborder. In the mammalian branch, the Tylopoda are classified together with the Ruminantia and Suiforms within the order Artiodactyla. This classification is also in agreement with recent molecular data. However, these antibodies have never been documented in species of these closely related taxonomic suborders (such as ovine, bovine, and porcine). Therefore, these heavy-chain antibodies should be considered as a unique occurrence, arising in Tylopoda (Nguyen et al., 2001, 2002).

In *C. dromedarius*, three IgG subtypes have been identified on the basis of their binding pattern to staphylococcal protein A and G. IgG_1 consists of two light (30 kDa) plus two heavy (50 kDa) chains, whereas IgG_2 and IgG_3 (heavy-chain antibodies) lack light chains and the CH_1 domain. IgG_2, a homodimer of 46 kDa chains, is characterized by an extended hinge region of Pro-X repeats (X = glutamic acid, glutamine or lysine). This long hinge could substitute for the CH_1 domain. IgG_2 binds only to protein A, whereas IgG_3, which consists of two chains of 43 kDa, after reduction binds to protein A and G (Hamers-Casterman et al., 1993; Lange et al., 2001).

A comparative study of old world camelids (*Camelus dromedarius* and *Camelus bactrianus*) and new world camelids (*Lama pacos*, *Lama glama* and *Lama vicugna*) showed that heavy-chain antibodies are abundant in the sera of all species examined and constituted up to 75% of the molecules binding to protein A. Furthermore it was found that the heavy-chain IgG antigen-binding domain of these antibodies consists of the variable domain of the heavy chain, referred to as VHH. VHHs have been proposed as valuable potential tools for biotechnology applications (Arbabi Ghahroudi et al., 1997). The size of these VHH fragments is reduced to a bare minimum (a single immunoglobulin domain) and the levels of recombinant expression and solubility are significantly higher than those of classical Fab or Fvs (non-covalently associated heterodimers of VH and VL domains). Several VHHs

have been raised in camels against different protein antigens and a few haptens. VHH from llama or camel have affinity constants for their protein antigens and are compatible to those of Fab or Fvs (Spinelli et al., 2000; van der Linden et al., 2000; Vranken et al., 2002).

Camels' immune responses against various antigens and their applications can be interesting. To our knowledge so far very little has been documented on camels' antibody responses against peptide antigens. MUC1 is a transmembrane molecule of which the major extracellular domain is composed of tandem repeat units of 20 amino acids (PDTRPAPGSTAPPAHGVTSA). The repeated units contain potential O-glycosylation sites represented by serine and threonine residues, which act as a scaffold for the attachment of O-glycans, resulting in the formation of a highly glycosylated extended repetitive structure (Qi et al., 2001). In breast, ovarian, lung, prostate, colon, and pancreatic cancer tissues, not only is MUC1 overexpressed, but the core protein is also aberrantly glycosylated, making the tumor-associated mucin antigenically distinct from the normal mucin (Taylor-Papadimitriou et al., 2000; Xing et al., 1992). Epitope mapping tests have been performed for antibody reactivity against overlapping synthetic peptides, and results were largely consistent among different groups. The dominant feature of epitopes within the MUC1 protein core is the presence, in full or part, of the hydrophilic sequence of PDTRPAP (Price, 1998). Therefore, rapid and sensitive screening methods for the detection of this molecule and their efficient targeting for the immunotherapy of cancer are highly essential. However the use of polyclonal and murine monoclonal antibodies has not been successful in immunotherapy of cancer target as initially expected due to their high molecular weight and structural immunogenicity. To this end the use of recombinant preparation of immunoglobulins or fragmented antibodies was preferred.

There are some reports on the generation of recombinant single chain variable domain fragment (scFv) antibody specific for tandem repeat region of MUC1 (Heuser et al., 2003; Kandilogiannaki et al., 2001). However these scFv-based constructs exhibit several serious shortcomings, for example; the tendency to form aggregates due to the presence of an oligopeptide linker, susceptibility of linkers to proteolytic cleavage, the unfolding of the antibody constructs, the immunogenicity and poor penetration of solid tumors by these antibodies (Muyldermans, 2001). In recent years attempts were made to solve these problems by humanizing the single chain antibody and by selection of an improved expression system. However the high costs for downstream processing (comprising the release of the fragments from the bacterial cells, unfolding/refolding procedures, extensive purification and detoxification of products), as well as the fact that engineered antibodies would not be as likely to elicit the same type of host immune responses that would be produced by

heterologous antibodies, are known to limit the use of these antibodies for immunotherapy. However naturally occurring heavy chain antibodies with close homology to human VH fragments, may open a new perspective of accelerated, sensitive, and very stable targeting vehicles especially for cancer therapy.

The main object of this study was to examine if such antibodies are produced in *C. dromedarius* and *C. bacterianus* against the MUC1 related peptide which is believed to recognize tandem repeat region and suitable for targeting this long known tumor marker. The natural or clinical origin such as; HMFG, D-HMFG, homogenized cancerous breast tissue and native MUC1 purified from ascitic fluid of a patient suffering from small cell lung carcinoma was also investigated.

MATERIALS AND METHODS

Two synthetic mucin peptides (TSA-P1-24 = TSAPDTRPAPGSTAPPAHGVTSAPDTR and A-P1-15 = APDTRPAPGSTAPPAH), corresponding to the mucin core protein, which was chemically conjugated to bovine serum albumin (BSA) by reaction with glutaraldehyde, were purchased from Q. Biogene, Co. (France). All other chemicals and reagents were analytical grade and purchased from Sigma Chemical Co. (USA).

Preparation of Tissue and Ascitic Fluid Samples

Fresh biopsies from malignant breast and pancreatic tissues and ascitic fluid of small cell lung cancer along with pathological reports were kindly provided by the Cancer Institute, I.K. Hospital, Tehran, Iran. Tissues were homogenized in liquid nitrogen using a mortar and pestle in PBS (20 mM, pH = 7), containing phenylmethylsulfonyl fluoride. The resulting suspension was centrifuged at 40,000 $\times$ *g* for 20 minutes at 4°C. The supernatant was then used for immunization of animals. An aliquot of the same antigen was used to recognize the antibody binding activity.

Cell Culture

Human breast cancerous cell lines (BT-20, BT-474, MDA-MB-361, MDA-MB-453, MDA-MB-468, MCF-7, T-47D, SK-BR-3, and Mel III), human colon cancer cell lines (HT 29, HT 29/219, SW 48, and SW 742), human ovary cancer cell lines (OVCAR-3, SK-OV-3, OVC1-PI 32 and A2780 CP) were used as the source of MUC1-positive expressing cell lines whereas a Hamster cell line (CHO) was used as a negative control.

Antigen Preparation

Fresh mature milk and colostrum samples from healthy mothers were collected. Fat globules were recovered by centrifuging at 40,000 × *g* for 60 minutes at 5°C. The fat globule fraction was collected and washed three times in saline, and then subjected to one cycle of freezing and thawing. The cell fraction containing membranes was collected by suspending the ruptured globules in warm normal saline and centrifuged at 40,000 × *g* for 90 minutes at 5°C. The pellet was recovered and subjected to two re-suspension-centrifugation cycles as described by Kandilogiannaki et al. (2001). For the preparation of defatted HMFG, the washed cream fraction of human milk was extracted twice with two volumes of chloroform and twice with 1 volume of ether, and lyophilized according to the method of Heuser et al. (2003).

A suspension of HMFG (10 mg/ml) was prepared by adding sodium cholate (2% w/w), urea (8 M), and 2-mercaptoethanol (2-ME) (1% v/v). The solubilized sample was centrifuged at 10,000 × *g* for 30 minutes at 15°C and the clear aqueous portion was recovered (Keenan et al., 1970).

Preparation of chemically deglycosylated HMFG (D-HMFG) which closely resembles cancerous MUC1 (Ceriani et al., 1977; Shimizu and Yamauchi, 1982), was performed by incubating an extensively dried sample (10 mg) in 400 μl trifluromethanesulfonic acid for 2 hours at 0°C, followed by neutralization with 1.5 ml of pyridine/water (3:2 V/V) at −20°C and extensively dialyzed against phosphate buffer (20 mM, pH 7) according to the procedure described by Hanisch et al. (1996). An aliquot of the sample was analyzed by SDS-PAGE. This assay was performed on 5% gel as described by Hames and Rickwood (1990).

Immunization of Camels

Two young adult male camels (*C. dromedarius* and *C. bactrianus*; purchased from a local cattleman) were immunized against MUC1 antigens (cancerous tissue homogenate, prepared as explained under materials and methods section, and a synthetic mucin peptide conjugated to BSA). Camels were injected intramuscularly with 1 ml of homogenized tissues equal to 12 mg tissue in Freund's complete adjuvant. Booster doses were given every 2–3 weeks with 7 mg of homogenized tissues and 250 μg of TSA-P1-24–BSA in a water-in-oil emulsion (van der Linden et al., 2000). Antiserum titration was started 1 week after the 5th injection. Blood was collected from the vena jugularis and serum was separated and stored at −70°C for future use.

Titration Assays

To estimate the titer of antibodies for each bleeding a titration curve was drawn following the procedure briefly explained as follows. One μg of synthetic

mucin peptide conjugated to BSA (TSA-P1-24–BSA) was coated onto the wells of micro titer plates and incubated at 37°C overnight. An equal amount of BSA was used as a negative control. Wells were washed, blocked with 1% BSA and filled with different dilutions of sera. In each experiment, normal camel serum at a dilution of 1:600 was used for non-specific binding (NSB). The plates were incubated at 37°C for 3 hours, washed, rabbit anti-camel–HRP added, and incubated at 37°C for 75 minutes. At the end of incubation period, wells were washed, 100 μl of 3,3′,5,5′-tetramethylbenzidine(TMB) added as substrate, and incubated for 10 minutes. The enzyme reaction was terminated with 50 μl of a 1 N sulfuric acid solution and color development was measured at 450 nm.

Precipitation of Camel Immunoglobulins

The globulin fraction of camels' antiserum was precipitated with 26% ammonium sulphate (w/v) and dialyzed against PBS (100 mM, pH 7). Alternatively, antiserum was precipitated by polyethylene glycol 6000 according to Polson et al. (1985).

Serum Fractionation

Serum was fractionated using protein G and protein A affinity chromatography (Hitrap, Pharmacia, Sweden). In order to find out the contribution of each of the three subclasses of IgG to the titer of the antiserum, the *C. dromedarius* and *C. bactrianus* sera were separated on protein G and protein A according to the procedure of Hamers-Casterman et al. (1993) with a minor modification. Briefly, 5 ml of normal and immunized camel sera were separately dialyzed against 10 mM phosphate buffer, pH 7.0 and then applied to the protein G column and equilibrated with the same buffer. An acidic buffer containing 0.15 M NaCl and 0.58% acetic acid, pH 3.5 first eluted IgG3. Subsequently, IgG_1 was eluted from the same column using 0.1 M glycine-HCl buffer pH 2.7. The protein A affinity column was used to purify the IgG_2 fraction which was not adsorbed on the protein G column. IgG_2 was eluted from this column with a buffer containing 0.15 M NaCl and 0.58% acetic acid, pH 4.5. The eluate was immediately neutralized with 100 mM Tris (pH 9.5), dialyzed against phosphate buffer (100 mM, pH 7) and stored at − 20°C. The protein contents of the affinity-purified fractions were estimated by modified Bradford protein assay based on the Ausubel et al. procedure (1999).

Characterization and Comparison of the Antibody Subclasses

In order to characterize the fractions purity and subclass, different fractions of IgG_1, IgG_2 and IgG_3 from camels were subjected to 12%

SDS-PAGE in the presence or absence of 2-ME (Hamers-Casterman et al., 1993). Protein bands were visualized by staining with coomassie prior to scanning. Molecular weight (MW) of the IgG subclasses was determined with the help of a calibration curve according to Hanisch et al. (1996).

Purification of Anti-Peptide Antibodies

The anti-peptide antibody was purified from whole antiserum using affinity chromatography columns. The columns were prepared by coupling of 5 mg of BSA to 0.4 gr of cyanogen bromide-activated Sepharose (Pharmacia) and 7 mg of TSA-P1-24–BSA to 0.6 gr of cyanogen bromide-activated Sepharose (Pharmacia) according to the manufacturer's recipe. The extent of coupling was found to be 1.5 mg of BSA to 1 ml of gel and 2 mg of TSA-P1-24–BSA to 2 ml of gel as judged by protein levels in the supernatant of the overlying gel.

Five ml of antiserum from *C. dromedarius* and *C. bactrianus* were separately loaded over the BSA column. The flow through was subsequently loaded onto a TSA-P1-24–BSA column to recover the specific anti-peptide antibodies. This was eluted with 100 mM glycine-HCl buffer, pH 2.7 from TSA-P1-24–BSA column. The eluent was immediately neutralized with Tris buffer (100 mM, pH 9.5) and dialyzed against phosphate buffer (10 mM, pH 7.0).

Affinity of Anti-Peptide Antibodies

Affinity of anti-peptide antibodies towards the antigen (synthetic peptides) was determined according to the procedure of Beatty et al. (1987). Micro titer plates were coated with two different concentrations (1 and 10 μg/ml) of peptide-BSA. An equal amount of BSA was coated and considered as negative control. Wells were blocked with 1% BSA and incubated for 2 hours with various concentrations (1, 2, 3, 4 nM) of antibodies, washed and incubated with diluted anti-camel-HRP (1:2000) for 75 minutes. The amount of bound enzyme activity was determined and the association constant (Ka) of antibodies to the peptide was calculated using the following mathematical expressions.

$$[Ag]/[Ag'] = n$$

$$K_a = n - 1/2(n[Ab'] - [Ab])$$

Fractionation of Anti-Peptide Antibodies

Different IgG subclasses of specific anti-peptide antibodies from *C. dromedarius* and *C. bactrianus* were separated using differential adsorption

on protein G and A. The protein content of the fractions was quantified by means of a modified Bradford protein assay (Ausubel et al., 1999).

ELISA

Three types of ELISA procedures were developed to determine the specificity and reactivity of the specific anti-peptide antibodies of *C. dromedarius* and *C. bactrianus*. These procedures are described below:

Rabbit Anti-Camel IgG Labeled-HRP Used as Tracer

Anti-camel IgG fractions were prepared and purified following the procedure described by Levy and Sober (1960). Immunoglobulin fractions purified from antisera were conjugated to HRP following a simplified $NaIO_4$ method (Tijssen, 1992) A suitable concentration of enzyme-conjugate was selected based on a titration assay.

To detect antisera and IgG fractions, two synthetic mucin peptides (A-P1-15 and TSA-P1-24) conjugated to BSA (1 μg/well of micro titer plates) were coated in micro titer plates at 37°C overnight. Whenever indicated, BSA alone was used in these assays and considered as a negative control. The contents of the wells were emptied, washed, and blocked for 1 hour at 37°C with PBS buffer containing 1% BSA. At the end of incubation period, wells were washed and different dilutions of sera, purified immunoglobulins and IgG fractions were added. In each experiment normal camel serum (1:600 dilution) or PBS were used as an index of NSB. Microplates were incubated at 37°C for 3 hours, washed, rabbit anti-camel–HRP was added, and incubated again at 37°C for 75 minutes. The rest of the procedure was performed as described above.

ELISA Using Purified Antigens

In the second type of assay, MUC1 of natural and clinical origin was purified and used as a reacting antigen.

1. HMFG or D-HMFG in PBS (10 mM, pH 7.4) containing 0.03% Triton X100 to give a final concentration of 1 μg/well was added to each well. The plate was allowed to dry overnight in a warm chamber and then blocked for 1 h at 37°C. The rest of the experiment was carried out as explained above.
2. The native cancer MUC1 from ascitic fluid of a patient with aggressive small-cell lung carcinoma and metastasis to the peritoneum was purified using an antibody-Sepharose affinity (immunoaffinity) column as follows: The immunoaffinity column was prepared by coupling two mg of the camel anti-peptide antibodies (obtained by peptide coupled affinity chromatography purification as described above) to cyanogen bromide-activated

Sepharose. Using this column, the MUC1 was eluted with acidic pH. The antigen obtained in this procedure was characterized using a murine monoclonal antibody (PR81) against the tandem repeat region of MUC1 (Paknejad et al., 2003). For this purpose, wells of micro titer plates were coated with 100 μl of different dilutions of purified antigen in PBS (20 mM, pH 7) overnight at 37°C and rest of the experiment was performed as described above.

Competition Assays

In this set of experiments in order to characterize the antibodies, several competition assays were performed. Specific anti-peptide antibodies of *C. dromedarius* and *C. bactrianus* were incubated with TSA-P1-24–BSA, A-P1-15–BSA, HMFG, D-HMFG, and purified cancer MUC1 at increasing concentrations (0–15,000 ng/ml) for 3 h at 37°C.

An irrelevant peptide with 14 amino acid residues, IRP1-14 (LEEKKG-NYVVTDHC) was used as negative control. The lowest antibody concentrations to give 50–70% maximum binding in each binding assays was first obtained by using checkerboard assay. Based on this, a concentration of 200 ng/ml was selected for the competitive binding assays of TSA-P1-24-BSA. Duplicate reactions were placed in wells precoated with 1 μg antigen per well and the bound antibody was detected as explained for the peptide-binding assay. The percentage inhibition rate was calculated from the optical density (OD) values in different ELISA assays, in relation to those of maximal binding (0% inhibition) and those of background binding (100% inhibition) of the respective antibodies (i.e., [1-OD sample/(OD max-OD background)] $\times$ 100%).

Immunocytochemistry

The reactivity of specific anti-peptide antibodies with human breast cancer cell lines (BT-20, BT-474, MDA-MB-361, MDA-MB-453, MDA-MB-468, MCF-7, T-47D, SK-BR-3, and Mel III), human colon cancer cell lines (HT 29, HT 29/219, SW 48 and SW 742), human ovary cancerous cell lines(OVCAR-3, SK-OV-3, OVC1-PI 32 and A2780 CP) and a Hamster cell line (CHO) as a negative control, were examined. Each cell line was grown on 96-well microtiter plates in RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in sterile 96-well culture plates. The cells were left to grow until being confluent. The supernatant was removed and the remaining medium was then air-dried, followed by addition of 100 μl a 5% H_2O_2 solution in PBS and incubation for 5 minutes. At the end of incubation period, wells were emptied, three times washed with PBS and blocked with 5% BSA. Anti-peptide antibodies at a

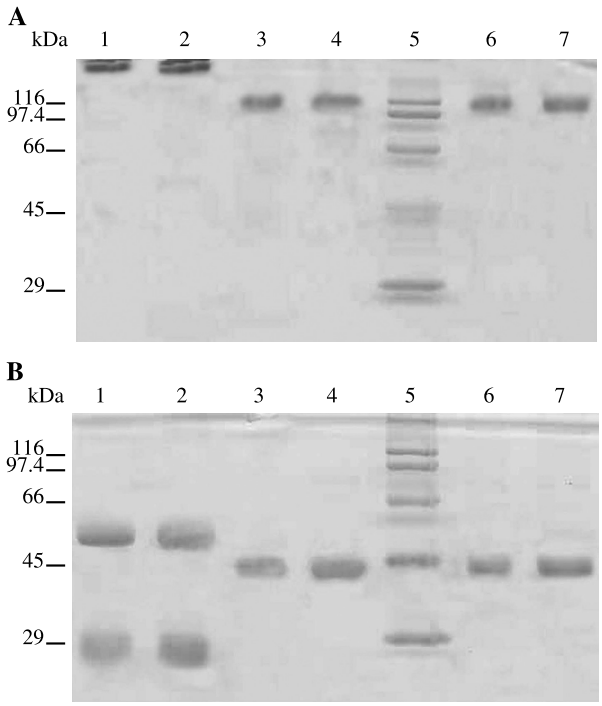

Figure 1: 12% SDS-PAGE, coomassie stain. (A) In the absence of 2-ME. 1: IgG_1 of *C. dromedarius*, 2: IgG_1 of *C. bactrianus*, 3: IgG_2 of *C. dromedarius*, 4: IgG_2 of *C. bactrianus*, 5: MW marker, 6: IgG_3 of *C. dromedarius*, 7: IgG_3 of *C. bactrianus*. (B) In the presence of 2-ME. 1: heavy chains and light chains of IgG_1 of *C. dromedarius*, 2: heavy chains and light chains of IgG_1 of *C. bactrianus*, 3: heavy chains of IgG_2 of *C. dromedarius*, 4: heavy chains of IgG_2 of *C. bactrianus*, 5: MW marker, 6: heavy chains of IgG_3 of *C. dromedarius*, 7: heavy chains of IgG_3 of *C. bactrianus*.

concentration of 0.5 μg/well were then added to each well (purified immunoglobulin obtained from normal camels was used as NSB) and processed as explained previously.

Western Blotting

HMFG, D-HMFG, homogenized cancerous and normal breast tissues, and purified cancerous MUC1 were subjected to 5% SDS-PAGE and transferred to nitrocellulose paper (0.45 μm pore size). Blots were blocked with 1.2% BSA in PBS containing 0.02% Tween-20 at room temperature for 60 min. Subsequently, the blots were incubated for 120 min with different purified sera and anti-peptide antibodies. Bound antibodies were detected by using polyclonal rabbit anti-camel IgG and sheep anti-rabbit IgG–HRP and developed with 3,3′-diaminobenzidine (Ausubel et al., 1999).

Table 1: Percentages of different protein A/G fractions found in serums of *C. dromedarius* and *C. bactrianus*.

	Fractions (%)			
Camel species	**IgG_1**	**IgG_2**	**IgG_3**	**Total IgG (mg/ml)**
C. dromedarius	25–30	25–30	35–45	8–12
C. bactrianus	25–30	25–35	40–45	8–12

RESULTS

Fractionation of IgG Subclasses

Serum samples from three non-immunized *C. dromedarius* and two non-immunized *C. bactrianus* were fractionated into heavy-chain and conventional IgG antibodies (Figure 1). Two purification methods were performed using ammonium sulphate and polyethylene glycol 6000 and the results were compared (data not shown). As shown in Figure 1 there was no difference in serum protein fractionations between *C. dromedarius* and *C. bactrianus*. The advantage of using polyethylene glycol 6000 for purification is that, the purified protein does not become contaminated with BSA. The conventional *C. bactrianus* IgG antibodies found in the G1 fraction designated as "IgG_1" according to Hamers-Casterman et al. (1993), were identical to those of *C. dromedarius*. In both the camels, this fraction (IgG_1) contained a molecule of approximately 160–170 kDa (Figure 1A, lanes 1 and 2) which upon reduction, yielded 50 kDa heavy chains and 30 kDa light chains (IgG_1 fraction bound to protein A and protein G, eluted in pH 2.7) (Figure 1B, lanes 1 and 2). The two other immunoglobulin fractions contained molecules of approximately 100 kDa (IgG_2 in Figure 1A, lanes 3 and 4 and IgG_3 in Figure 1A, lanes 6 and 7), which upon reduction yield only heavy chains, having a molecular masses of about 46 kDa (IgG_2 fraction binding only to protein A, eluted at pH 3, Figure 1B, lanes 3 and 4) and approximately 43 kDa (IgG_3 fraction binding to protein A and protein G, eluted in pH 3.5, Figure 1B, lanes 6 and 7).

These data clearly show that the two IgG subclasses lack any light chains. Fractionation results for serum obtained from *C. bactrianus* compared with *C. dromedarius* showed that there is no difference between the immunoglobulins of the old world camels with respect to MW. It was found that the total amount of IgG in serum samples of old world camels varied between 8–12 mg/ml. In both camels, most of the IgGs consisted of heavy chain IgG antibodies (70–75%) and the conventional hetero-tetrameric IgG were clearly less abundant (25–30%). Estimation of the relative amounts of the isolated camels IgG

Table 2: ELISA results of crude camels polyclonal antibodies at OD_{450} nm for various antigens using rabbit anti-camel–HRP as tracer.

	C. dromedarius			*C. bactrianus*			
Antigens (1 μg/well)	**IgG_1**	**IgG_2**	**IgG_3**	**IgG_1**	**IgG_2**	**IgG_3**	**NSB[a]**
BSA	0.60	0.54	0.60	0.60	0.55	0.60	0.56
A-P1-15–BSA	1.96	1.55	1.98	1.90	1.28	1.88	0.61
TSA-P1-24–BSA	2.27	1.48	2.51	2.20	1.25	2.30	0.60
HMFG	1.96	1.62	1.90	1.68	1.40	1.70	0.62
D-HMFG	2.10	1.90	2.10	1.80	1.70	1.80	0.61

All results are expressed in term of OD.
[a]Purified normal camels' immunoglobulin was used as the NSB index.

subclasses revealed that the IgG_2 fraction is the least abundant immunoglobulin (Table 1).

Camels' Immune Response

The immune response was followed by analysis of whole serum and purified serum (precipitation methods) with antigen-specific ELISAs. A significant antibody response was detected 120 days after the first immunization in both camels. In order to investigate whether heavy-chain IgG subclasses were induced, sera were fractionated by protein G and A affinity chromatography. Antigen binding activity of each IgG subclass was detected using rabbit anti-camel–HRP. In this assay, camel immunoglobulins purified

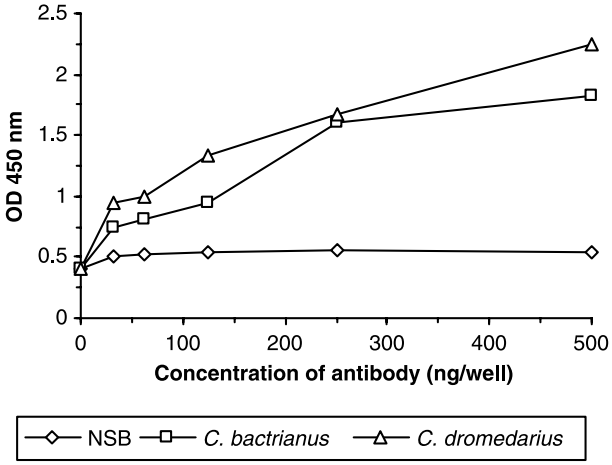

Figure 2: Reactivity of purified anti-peptide antibodies with A-P1-15–BSA. NSB was determined using purified normal camel immunoglobulin.

Table 3: Percentages of different IgG subclasses found in anti-peptide antibodies of *C. dromedarius* and *C. bactrianus*.

Camel species	Fractions (%)			Total anti-peptide IgG (μg/ml)
	IgG_1	IgG_2	IgG_3	
C. dromedarius	25.44	32.50	42.04	80
C. bactrianus	26.30	31.46	42.16	70

from normal camel were used as NSB. The results indicated that suitable titers of conventional and heavy-chain antibodies were obtained (Table 2).

Purification and Reactivity of Specific Anti-Peptide Antibodies

Approximately 400 μg of pure anti-peptide IgG was obtained out of 5 ml of serum of both immunized camels loaded on affinity chromatography columns. Affinity purified antibodies in an ELISA procedure showed a high immunoreactivity (Figure 2). The purity of antibodies eluted from the columns was evaluated by SDS-PAGE. All the IgG subclasses were present in this fraction (data not shown).

A significant difference between the ODs of purified anti-peptide antibodies and purified normal camel immunoglobulin indicated that camels were successfully immunized against the tandem repeat region of MUC1. The results of affinity determinations showed that K_a of the specific polyclonal

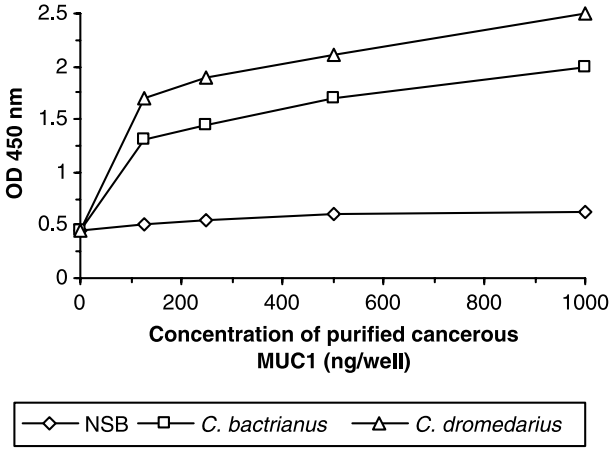

Figure 3: Reactivity of specific anti-peptide antibodies with cancer MUC1 (purified from ascitic fluid). Concentration of antibodies: 200 ng/well. NSB was determined using purified normal camel immunoglobulin. All assays were performed in duplicate.

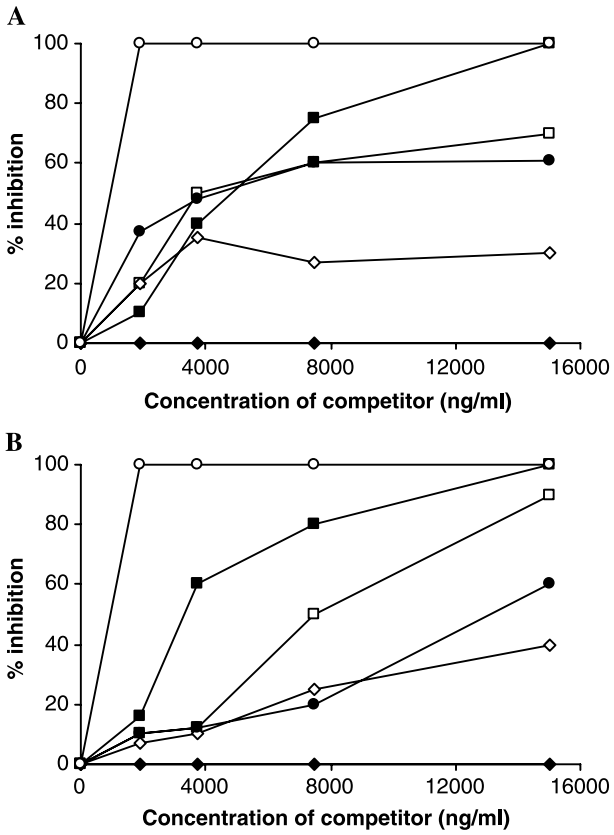

Figure 4: Percentage inhibition of the binding of the specific anti-peptide antibodies in *C. dromedarius* (A) and *C. bactrianus* (B) to TSA-P1-24–BSA by purified cancer MUC1(○), TSA-P1-24–BSA (■), A-P1-15–BSA (□), D-HMFG (●), HMFG (◇), IRP1-14–BSA (◆), at increasing concentrations (0–15,000 ng/ml) in an ELISA assay.

anti-peptide antibodies was around 7×10^{10} M^{-1} and 1.4×10^{10} M^{-1} in *C. dromedarius* and *C. bactrianus*, respectively. Table 3 shows the relative amounts of the isolated specific anti-peptide antibody subclasses. It is obvious the IgG_3 subclass contained higher amounts of anti-peptide immunoglobulins as compared to other subclasses.

Purification and Reactivity of Native Cancer MUC1 of Ascitic Fluid

Affinity purification of MUC1 from 50 ml of ascitic fluid yielded approximately 150 μg native cancer MUC1. Figure 3 shows the binding behavior of specific anti-peptide antibodies to purified cancer MUC1 of ascitic fluid.

Competitive Binding Assays of Antibodies

Figure 4 shows the rate of binding inhibition of the camel anti-peptide antibodies. Binding of both anti-peptide antibodies to TSA-P1-24–BSA was totally inhibited in presence of purified cancer MUC1, whereas IRP1-14–BSA has little inhibitory activity. Other inhibitors showed variable inhibition activities (Figure 5).

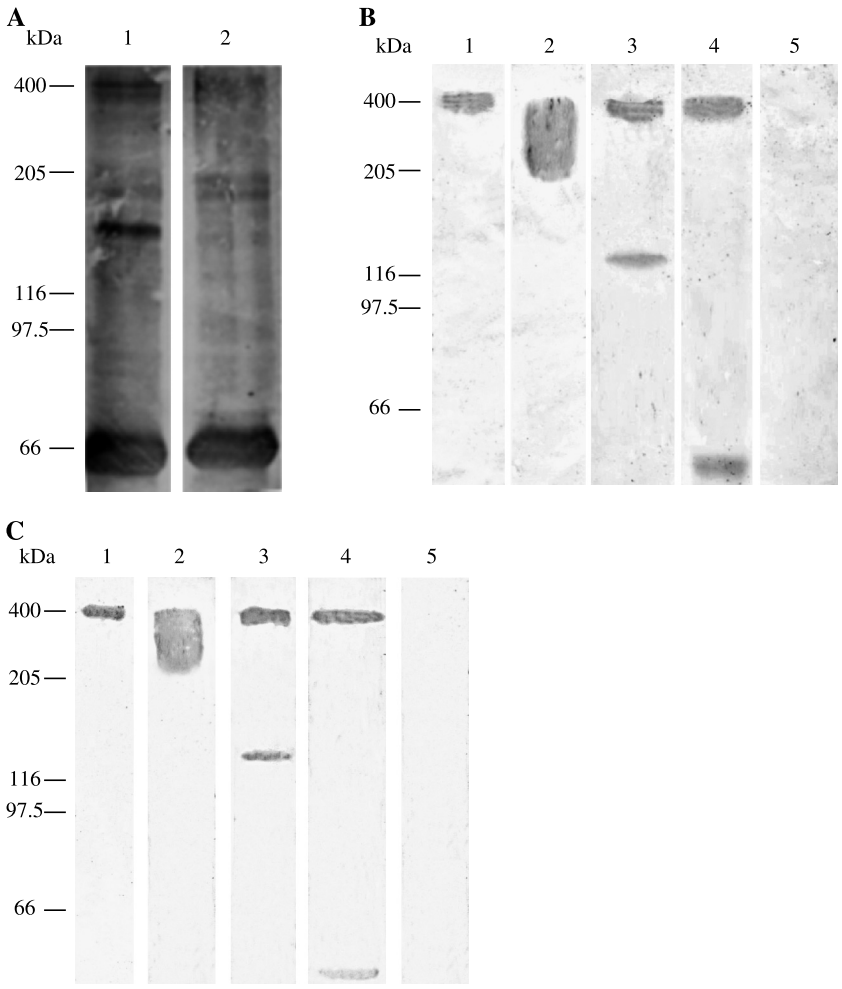

Figure 5: Immunoblotting using: (A) immunoglobulins fractions of *C. dromedarius* or *C. bactrianus*. 1: homogenized breast cancer tissue, 2: homogenized normal breast tissue. (B) Purified anti-peptide antibodies of *C. dromedarius* and (C) purified anti-peptide antibodies of *C. bactrianus*. 1: HMFG, 2: D-HMFG, 3: purified cancer MUC1 of ascitic fluid, 4: homogenized breast cancer tissue, 5: homogenized normal breast tissue.

Table 4: Immunocytochemistry results of anti-peptide antibody performed with different cell lines.

Cell lines	*C. dromedarius*	*C. bactrianus*	NSB[a]
CHO	0.42	0.40	0.34
BT-20	1.34	1.34	0.36
BT-474	1.33	1.20	0.38
MDA-MB-361	0.91	1.09	0.33
MDA-MB-453	0.89	0.91	0.35
MDA-MB-468	1.08	1.00	0.39
MCF-7	1.40	1.30	0.37
T47D	1.27	1.35	0.35
SK-BR-3	0.91	0.89	0.37
Mel III	1.21	1.34	0.40
HT29	1.40	1.41	0.35
HT29/219	1.35	1.43	0.43
SW 48	1.34	1.43	0.35
SW 742	1.01	1.00	0.38
OVCAR-3	0.77	0.9	0.36
SK-OV-3	1.02	1.22	0.33
OVC1-PI 32	0.79	0.87	0.34
A 2780 CP	0.99	0.90	0.38

All results are expressed in term of OD.
All assays were performed in duplicate and the results are average of binding activity of heavy chain antibody towards each cell line.
[a]Purified normal camels' immunoglobulin was used as the NSB index.

Immunocytochemistry

Table 4 shows the results of experiments carried out to determine the binding of specific anti-peptide antibodies to cancerous cell lines and strains with human origin. In this experiment, CHO cells of hamster origin were used as a negative control. It was observed that these antibodies reacted specifically with human cancerous cell lines. Of considerable interest is the observation that both of these antibodies showed a negative reaction towards CHO cells.

Reactivity of Camel Serum Immunoglobulin and Anti-Peptide Antibodies to MUC1

In order to confirm the data obtained in ELISA procedure, a Western blotting experiment was performed in which purified cancerous MUC1 of ascitic fluid, HMFG, D-HMFG, homogenized cancerous and normal tissues were separated on 5% SDS-PAGE and blotted on nitrocellulose paper. Membranes were developed with the appropriate dilutions prepared from immunoglobulins fractions of *C. dromedarius* or *C. bactrianus* (A), purified anti-peptide antibodies of *C. dromedarius* (B) and *C. bactrianus* (C) (Figure 5). Under these circumstances, with homogenized breast cancer and normal breast tissues, the recognition pattern for purified serum immunoglobulins

(A) and specific anti-peptide antibodies (B and C) varied profoundly. Serum antibodies (A) bound to various proteins were visualized by coomassie staining of SDS-PAGE gel of the cancerous and normal tissue preparations (results not shown). In contrast, the specific anti-peptide antibodies recognized the bands of approximately 400 kDa in homogenized cancerous tissues (lane 4), but failed to react with homogenized normal tissues (lane 5). For purified native cancer MUC1 of ascitic fluid a protein band of about 400 kDa was recognized by the anti-peptide antibodies (lane 3). In lane 2, one broad band with a molecular mass of 200–400 kDa was found which indicates the presence of a MUC1-related peptide motif and partially deglycosylation of the core protein of MUC1 in preparations of D-HMFG from HMFG. For crude HMFG, at higher concentrations, a band is observed of about 400 kDa (lane 1).

DISCUSSION

Antibodies are used as important biopharmaceutical agents in immunotherapy and in scanning of cancer tissue .However the most important obstacle in wide use and application of these reagents is the problem of size and protein heterology. Therefore the main attention of those working in the field of immunotherapy would be to address those two important problems. If solved, these reagents may well be used as magic bullets in targeting the desired cells without much adverse effects as far as has been observed to date.

Our current knowledge of immunology indicates that in addition to antibodies consisting of pairs of H-chains and L-chains, which are present in all vertebrates, a set of unconventional antibodies devoid of L-chains in disordered immunoglobulins somatically generated can also exist, which cause heavy chain disease (Franklin et al., 1964). In 1993, the first *bona fide* antibodies devoid of L-chains in their natural structure were described as additional IgG classes in all present-day camelidae (Hamers-Casterman et al., 1993). All H-chains of heavy-chain antibodies of camelids lack the entire CH_1 domain, but their variable domain is intact and the hinge is present as well. The CH_2 and CH_3 domains harbor the effector functions. In addition, these parts also contain the signals for Fc receptor, complement C1q, and protein A binding sites (Nguyen et al., 2001). It seems, therefore, that the heavy-chain antibodies will exert the conventional effector functions. Hence, these antibodies appear to be fully functional and an integral part of camels' and llamas' immune systems.

Following the initial observation by Hamers-Casterman et al. (1993), a number of other studies showed that camels' heavy chain antibodies repertoire, when purified, displays a recognition pattern different from that of conventional IgGs. In one of these studies it was found that antigen-specific serum titers obtained for protein antigens (60–80 kDa) are consistently higher

than those obtained with haptens and that they were preferentially bound by heavy chain antibodies (van der Linden et al., 2000). However others observed that, immunization of a lama with hapten coupled to a BSA carrier leads to a higher relative proportion and binding activity of heavy chain antibodies against the hapten as compared to the carrier (Lange et al., 2001).

In this study anti-MUC1 antibodies were raised against a synthetic peptide that consists of the tandem repeat region of MUC1. Based on our results it is assumed that immunization of old world camels with a synthetic peptide and tissue extract could result in high yield heavy chain antibodies. This fact is justified firstly by the presence of high amounts of heavy chain antibodies (IgG_2 and IgG_3) as compared to conventional IgG in camel sera and secondly that these antibodies showed greater affinities to the peptide antigens due to its molecular structure. Despite the high yield antibodies obtained in these studies, it is premature to draw a general conclusion on the behavior of heavy chain antibodies towards a peptide antigen since only two camels were immunized.

The close evolutionary relationship between old world camels, was reflected in this study when sera samples from both camels were subjected to purification, fractionation and immunoassay protocols under similar experimental conditions. These results clearly showed that *C. bactrianus* as well as *C. dromedarius* possesses both kinds of heavy-chain antibodies (IgG_2 and IgG_3) as judged by affinity purification and antigen specific responses present in the purified heavy chain antibody fractions, with IgG_3, being the major constituent of IgGs (up to 45%) in both old world camels, having approximately 8–12 mg/ml, serum IgG.

Another aspect of camel antibody was its immense similarity to the human immunoglobulin and lower molecular mass (90–100 kD) as compared to normal immunoglobulins. Comparison of the human VH3 gene family (the largest member of human immunoglobulin VH repertoire) with the camelid VHH shows a high degree of homology as expected for genes of the same family (Muyldermans, 2001), suggesting the potential application of camel antibodies for targeting human tumor marker antigens.

In this context and in order to examine the application and behavior of heavy-chain antibodies, MUC1 (tumor-associated epitope on a high-MW glycoprotein molecule) was selected as the targeting factor (Girling et al., 1989). The expression of MUC1 was found to be dramatically increased in a large number of malignancies. Overexpression and differential glycosylation pattern of mucin molecules in breast, ovarian, lung, prostate, colon, and pancreatic cancer can result in different exposure patterns of the core protein. These epitopes (MUC1) are probably masked in the mucin produced by normal cells, however the epitope in the 20-amino acid tandem repeat sequence was found to be accessible in these types of cancer. Furthermore in the normal

glandular epithelial cells, MUC1 expression is limited to the apical surface bordering to lumen but in cancer cells which have lost polarity the mucin is expressed all over the surface, which is believed to affect tumor progression (Price, 1998; Taylor-Papadimitriou et al., 2000).

The behavior of *C. dromedarius* and *C. bactrianus* when challenged with a peptide of definite size and antigenic determinant was compared. In order to have a valid assessment of anti-peptide antibodies, it was important to examine their reaction with MUC1 of varying sources including HMFG, D-HMFG and cancer MUC1 purified from metastatic ascitic fluid in direct binding ELISA, inhibition ELISA, Western blotting and the reaction with cell lines in immunocytochemistry. Antigen specific responses appeared in the purified heavy chain antibodies in all cases for the tandem repeat region of MUC1 mucin.

Involvement of the tandem repeat region in antibody reaction was shown when the interactions were compared of whole antiserum and anti-peptide antibodies with homogenized cancer and normal tissues. These results indicated that the whole antiserum does react with whole tissue homogenate proteins without discrimination. However when these antibodies were purified using an immunoaffinity column, the resulting antibodies from both animals only reacted with the protein under investigation (around 400 kDa band) and could discriminate between normal and cancer tissues in Western blotting. The main reason for this finding would be that a synthetic peptide containing the tandem repeat region of MUC1 was used together with cancer tissue homogenate, to immunize the animal and the antibody obtained was purified against the same peptide sequence. This evidence shows that the antibody could specifically react with the core peptide that is perhaps masked in native protein and unmasked in the cancerous form of MUC1 mucin.

Using anti-MUC1 monoclonal antibody (PR81) (Paknejad et al., 2003) the specificity of polyclonal antibodies purified from camels' sera was further confirmed. Both antibodies exhibited similar reactions towards the TSA-P1-24 antigen and failed to react with an irrelevant peptide sequence, (IRP1-14). This further confirmed our initial assumption that anti-peptide antibody may be purified to the extent of mono-specificity, so that it can be used as a targeting reagent for recognition of the MUC1 tandem repeat appearing on the surface of malignant cells.

To our knowledge, this is the first report on the use of a limited peptide (the epitope in the tandem repeat of the core protein of the MUC1 mucin) and homogenized cancer tissues as immunogens for the production of heavy-chain antibodies in old world camels. Further studies may pave the way for the applications of these antibodies in the fields of medical diagnosis and targeted drug therapy.

ABBREVIATIONS

2-ME	2-mercaptoethanol
BSA	bovine serum albumin
CH_1, CH_2 and CH_3	constant heavy-chain domains
D-HMFG	Deglycosylated human milk fat globule membrane
ELISA	enzyme linked immunosorbant assay
Fab	light chain disulphide-bounded to a fragment of heavy chain containing the VH and CH_1 domains
Fvs	non-covalently associated heterodimers of VH and VL domains
HMFG	human milk fat globule membrane
HRP	horseradish peroxidase
Ig	Immunoglobulin(s)
MW	molecular weight
NSB	non-specific binding
OD	optical density
PBS	phosphate buffered saline
scFv	single chain variable domain fragment
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
VH	variable domain from the heavy chain of conventional antibodies
VHH	the VH fragment from a camel heavy chain antibody
VL	variable domain from the light chain of conventional antibodies

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