Research Article

Immunological methods to develop camelid anti-cancer

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Abstract

Background: The humoral immune response of the Camelidae is unique as these animals are the only known mammals that seem to possess functional homodimeric heavy-chain antibodies besides the classical heteromeric antibodies composed of heavy (H) and light (L) chains.

Objective: to assess the ability of camel IgGs to react much better to the tumor associated antigen (TAA) than human IgGs and independently from the haplotype-restriction of the TAA complexes with MHC molecules.

Materials and methods: The immunization of carcinoembryonic antigen (CEA) of two adult camels was carried out in the private farm at Sharjah – UAE for five weeks and then the specimens were collected and transferred to the central laboratory of the Sharjah University. The project was extended from April 2008 to April 2009. The specimens were fractionated by chromatography then were evaluated by using Elisa technique.

Results: The results of CEA immunization of adult camel and its calf was determined by using indirect ELISA test. The ELISA readings showed positive response with camelid serum, urine, milk and calf serum. Negative results showed with pre-immunized serum from camel and its calf and from blocking buffer.

Conclusions: This work succeeded in formulating TAA-specific highly reactive camelid IgG that are able to recognize only the cancerous cells and sparing the normal cells. These TAA-specific antibodies bound tightly to TAA antigens as well as to control carcino-embryonic antigen (CEA).

Key words: CEA, Camelid IgG, TAA, indirect ELISA, VHH

INTRODUCTION

The humoral immune response of the Camelidae is unique as these animals are the only known mammals that seem to possess functional homodimeric heavy-chain antibodies besides the classical heteromeric antibodies composed of heavy (H) and light (L) chains. By definition, the heavy-chain antibodies lack the L-chain, and it was noticed that their H-chain is devoid of the typical first constant domain (CH1) and contains a dedicated variable domain, referred to as VHH. The VHH exon is assembled from separate V–D–J gene segments. The recombined VHH region is subjected to somatic hypermutations; however, the timing and actual mechanism of the class switch from μ-isotype to the dedicated γ-isotype remains elusive. Interestingly, antigen-specific VHHs are easily retrieved after panning of a phage-displayed rearranged V-gene pool cloned from an immunised camelid (1, 2). These single-domain antigen binding entities possess a number of biophysical properties that offer particular advantages in various medical and biotechnological applications (3, 4). Cancer is a group of diseases in which cells are aggressive (grow and divide without respect to normal limits), invasive (invade and destroy adjacent tissues), and sometimes metastatic (spread to other locations in the body) (5). Cancer may affect people at all ages, even fetuses, but risk for the more common varieties tends to increase with age (6). Tumor antigens are useful in identifying tumor cells and are potential candidates for use in cancer therapy. Initially they were broadly classified into two categories based on their pattern of expression: Tumor-Specific Antigens (TSA), which are present only on tumor cells and not on any other cell and Tumor-Associated Antigens (TAA), which are present

on some tumor cells and also some normal cells. This classification, however, is imperfect because many antigens thought to be tumor-specific turned out to be expressed on some normal cells as well (7, 8). Oncofetal antigens are another important class of tumor antigens. Examples are α-fetoprotein (AFP) and carcinoembryonic antigen (CEA) (9). These proteins are normally produced in the early stages of embryonic development and disappear by the time the immune system is fully developed. Thus self-tolerance does not develop against these antigens (10).

In this project, we test a new hypothesis that is the ability of camel IgGs to react much better to the TAA than human IgGs and independently from the haplotype-restriction of the TAA complexes with MHC molecules. Since camelid subclass IgG2 proved to act as a potent enzyme neutralizer because the antigen binding site of the VHH is small, stable, tight, and convex in topography which enables it to get through the enzyme cleft. Alike, we assume that this advantage could be applied to a very similar extent on the contact of VHH with TAA enclaved within the MHC class I cleft (3, 11).

PATIENTS AND METHODS

Dromedary immunization of camel for certain antigen

One newly delivered camel (Camelus dromedarius) was injected with 10 mg to 50 mg of the formed immunogen (Carcinoembryonic antigen (CEA; Sigma-Aldrich) + Fruend's adjuvant; Sigma-Aldrich) at days 0, 7, 14, 21 and 28. This period can result in successful immunization. The camel was injected with CEA antigen and complete Fruend's adjuvant at 1st injection and completed with CEA antigen and incomplete Fruend's adjuvant for the subsequent injections. Serum, milk and urine were collected before the immunization and prior to each injection to follow-up the immune response against the immunogens. At day 35, anticoagulated blood was collected for lymphocytes isolation. Peripheral blood lymphocytes prepared with Unisep (WAK Chemie, Germany). Cells were counted and pellet aliquots of 5×106 cells stored at -80°C until further use (11, 12).

Fractionation of the CEA specific camelid IgGs

Four IgG subclasses were purified from the dromedary serum, milk and urine by differential absorption on Protein A and Protein G. The IgG1 subclass contains the conventional heterotetrameric antibodies composed of two light and two heavy chains, whereas IgG2a, IgG2b and IgG3 are the homodimeric heavy-chain antibodies, devoid of light chains (13, 14).

The prevalence of specific heavy-chain antibodies was confirmed following a separate approach in which total serum was incubated with the native antigen immobilized on Sepharose. The captured proteins thereafter were analyzed on SDS-PAGE.

IgG subclasses were obtained by successive affinity chromatography on 1 ml HiTrap Protein G and Protein A columns (Pharmacia). IgG3 and IgG1 were eluted from the Protein G column with an acetate buffer (pH 3.5) and a glycine- HCl buffer (pH 2.7), respectively. The flow-through was loaded on the Protein A column to recover two more fractions of heavy-chain antibodies, IgG2a and IgG2b were recovered with the acetate buffer at pH 4.5 and 3.5, respectively. The IgG protein concentrations were determined specrtophotometrically , assuming an 1% of 13.5 at 278 nm for all subclasses (15, 16). The conventional IgG1 antibodies was expected in the range of Mr 160 000 Da, huge amounts of heavy-chain antibodies with Mr of ~95 000 Da, whereas, IgG2 and IgG3 of dromedary were the monomeric heavy chains of (Mr 45 000 and 42 000 Da, respectively). Therefore, the protein band at molecular weight of 45,000 and 42,000 da was eluted as shown earlier for further ELISA validation of these IgG2 and IgG3 against TAA.

Measurement of the anti- CEA Ab response

The method of indirect ELISA used to measure the anti-CEA antigen as follows: Three microtiter plates were coated with the CEA antigen. The studied samples and controls were carried-out in duplicate. CEA was adsorbed onto a 96-microtiter plate in carbonate buffer (pH 9.6) overnight at 4°C. Bovine serum albumin (BSA; 67,000 M.W.) is often used as non-relevant protein to assess anti-CEA antibody titers. BSA was used as blocking buffer, 10 mg/mL in PBS/Tween-20 and 50 ul were added for 1 h at 37°C. Nonspecific binding sites on the microtiter plate were blocked by using a blocking buffer after coating the CEA antigen to the plate. After washing step, different serum specimens, withdrawn from the immunized camels and its baby were added onto the CEA-coated microtiter plates. These samples included serum, milk, urine of immunized camel and the serum of a camel baby. Immunized serum, milk, urine, and baby's serum were diluted 1:200 respectively, and 100 µl of each dilution were added on the coated microtiter plate to bind the adsorbed TAA for 2 hours at 37°C. Any CEA non-specific antibodies or unbound were washed away by washing step three times with phosphate buffer saline (PBS; BDH) containing 0.05% Tween-20 (BDH) before the next assay step. The negative control was divided into three categories to exclude all the misdiagnosis and cross reaction possibilities or the false positive and\or negative possibilities. Category (A) included serum from pre-immunized camel and category (B) included serum from

pre-immunization baby. However, category (C) included blocking buffer instead of serum. 100 µl of secondary antibody-enzyme conjugate (goat anti-human IgG (γ- chain specific) peroxidase conjugate) in 1:30 000 PBS/Tween-20 was used to bind the anti-CEA antibody for 1 hour at 37°C. After washing, the bound antibodies were detected by adding TMB substrate. Then the reaction was measured by using ELISA reader (Bioteck) at wavelength 450ηm. One immunization protocol, for example, may produce an antibody titer/ the higher the dilution factors, the stronger the polyclonal immune response (15).

Lyophilization

Fifteen milliliters samples of immunized camel milk were placed in ice cubes container and frizzed up to -70ºC. At appropriate time 5 frizzed milk cubes milk were placed in the lypholizer beakers and the latter were placed in lypholizer and ran with the sucking power to create a negative pressure and reduce temperature to -70ºC for 36 hrs (16).

Statistical analysis

Statistical analysis were conducted to describe different variables and parameters in research and to determine relationship with each other as well. The SPSS (2010) was used to effect of different actors in study parameters. The T-test is the comparative between means in this study. Means was described by their relative standard errors (SE) to indicate the variability of the data and the precision of estimated samples. Probability values of $P < 0.05$ and 0.01 were considered statistically significant.

RESULT

The results of CEA immunization of adult camel and its calf was determined by using indirect ELISA test. The ELISA readings showed positive response with camelid serum, urine, milk and calf serum. Negative results showed with pre-immunized serum from camel and its calf and from blocking buffer, Fig (1).

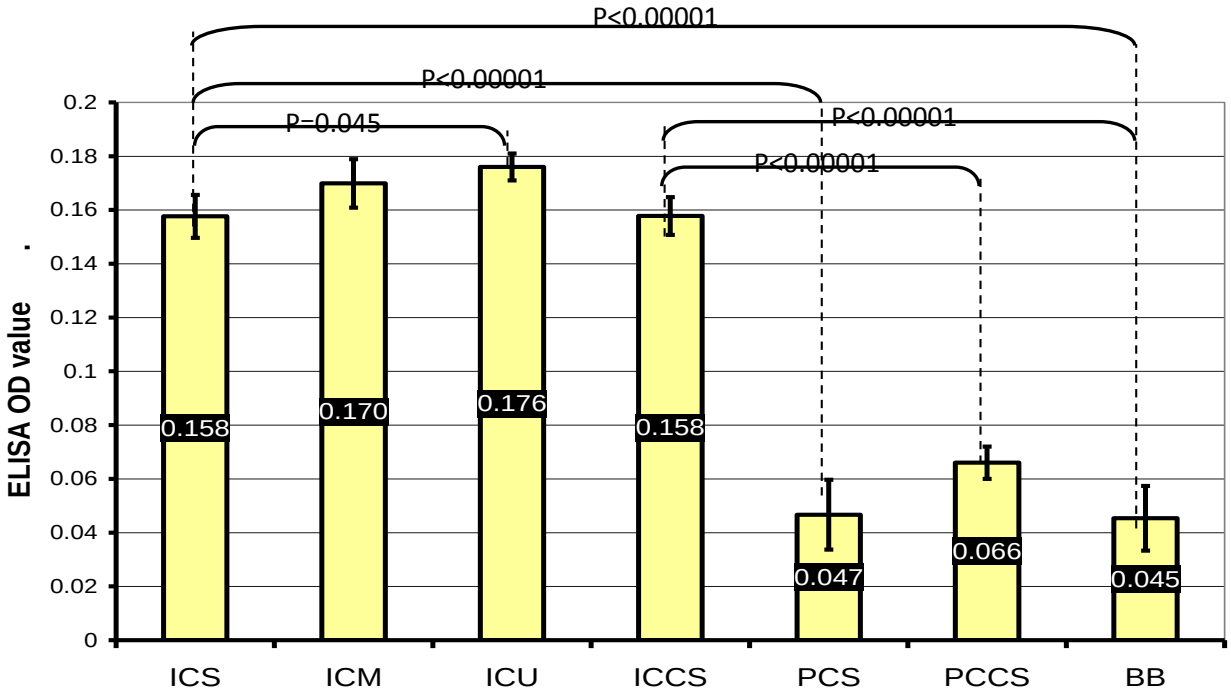

Figure 1. Statistical analysis of CEA immunization of adult camel and passive immunization to camel calf. The histogram showing the comparative differences in mean ±STEM of immunized camel serum (ICS), immunized camel milk (ICM), immunized camel urine (ICU), immunized camel calf serum (ICCS), pre-immunized camel serum (PCS), pre-immunized camel calf serum (PCCS), and negative control blocking buffer (BB). The assigned P values are the only significant differences in this figure.

Immunized camel serum is significantly lower than immunized camel urine (P<0.05, P=0.045). However, immunized camel serum is not significantly different from both immunized camel milk (P=0.117) and passive immunized camel calf serum (P=0.49). Moreover, immunized camel urine and immunized camel milk are not significantly different (P=0.3). The immunized camel serum and passive immunized camel calf serum are far significantly higher than pre-immunized camel serum and pre-immunized camel calf serum respectively (P<0.00001). Furthermore, immunized camel serum and passive immunized camel calf serum are far significantly higher than blocking buffer (P<0.00001). Meanwhile, No significant difference between blocking buffer, pre-immunized camel calf serum, and pre-immunized camel serum.

DISCUSSION

The immunization of CEA in both adult camel and passive immunization of camel calf turned out very successful in comparison with pre-immunized sera of the same animals. This proved that camel immune system is highly responsive to the positive control, namely, CEA and it has passed through udder feeding to the calf.

Furthermore, the research has also shown that the adult camel's urine contains higher anti-CEA IgG than adult camel's serum. The promising result of this phase is that the anti-CEA IgG antibodies were most abundant in urine where is the easiest fluid collected from the animal. Collecting urine does not need invasive injections, like in serum case, or restrict the yield period to the post-pregnancy time, like in milk case. However, to the best of our knowledge and according to the literature reviews this is the first time to find huge amount of immunoglobulin in urine whether from any animal or human. Therefore, this finding will make the urine of camel as important source of IgG that means the camelid urine becomes the cheapest valued immunetherpeutic solution and this is an interesting finding (2,16, 17).

Furthermore, Camel's milk contains higher amount of IgGs than that of serum but did not reach significance level.

On the other hand, the research has shown that the CEA-IgG presence in high rate in the calf serum that has udder feeding from the immunized camel and the significant breakthrough that there is no difference in the level of anti-CEA IgG in adult and calf sera. This finding confirmed there where a complete passive immunization of antibodies from mother to its calf occurred through milk feeding. Thus in the similar way it is now possible to immunize human or huge number of animal herd through-out simple milk feeding from immunized camel against any desired pathogenic antigen, therefore this significantly can cut the cost of expensive immunization of each individual animal and it can be accomplished also within a shorter period.

This finding represents a reflection on the behavior of camel towards immunization against TAA or any other antigen. Therefore, it is possible to use camel species as a targeted IgG antibodies factory for any desired antigen and easily can use urine, which is daily extracted, as source of this valuable cure. In addition, for more advancement in the figured out research, it is conceived in the second phase of this project to use clinically isolated and eluted TAA from different tumor biopsies or tumor cell lines to deploy camel's factories against these TAA. Furthermore, the top achievement, the preparation of endless in vitro source of the produced target IgG antibodies via molecular cloning of competent E. coli without using any more urine, serum, or milk of the camel. Nevertheless, camel urine, serum, and milk are considered a cheaper and simpler method, neutriceutical approach, for getting high quality, single domain, single chain camelid IgG against any desired pathogenic antigen.

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