Journal of Immunological Methods xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical note

Optimizing selection of large animals for antibody production by screening immune response to standard vaccines

Mary K. Thompson [a], Peter C. Fridy [a], Sarah Keegan [b], Brian T. Chait [c], David Fenyö [b], Michael P. Rout [a,*]

[a] Laboratory of Cellular and Structural Biology, The Rockefeller University, New York, NY, USA
[b] Center for Health Informatics and Bioinformatics, New York University School of Medicine, New York, NY, USA
[c] Laboratory of Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, New York, NY, USA

ARTICLE INFO

Article history:
Received 13 November 2015
Received in revised form 23 December 2015
Accepted 5 January 2016
Available online xxxx

Keywords:
Immunization
Vaccine
Antibody generation
Camelid antibody
Antigen

ABSTRACT

Antibodies made in large animals are integral to many biomedical research endeavors. Domesticated herd animals like goats, sheep, donkeys, horses and camelids all offer distinct advantages in antibody production. However, their cost of use is often prohibitive, especially where poor antigen response is commonplace; choosing a non-responsive animal can set a research program back or even prevent experiments from moving forward entirely. Over the course of production of antibodies from llamas, we found that some animals consistently produced a higher humoral antibody response than others, even to highly divergent antigens, as well as to their standard vaccines. Based on our initial data, we propose that these "high level responders" could be pre-selected by checking antibody titers against common vaccines given to domestic farm animals. Thus, time and money can be saved by reducing the chances of getting poor responding animals and minimizing the use of superfluous animals.

1. Introduction

Large animals have long held important roles in diverse areas of biomedical research. Small animals are much more common, due to their ease of use and the availability of genetic manipulation systems; but large animals including goats, sheep, horses, donkeys and camelids all have distinct advantages. Large animals tend to be better than murine models for diseases such as tuberculosis, influenza, asthma and Crohn's disease, among others (Conti et al., 2014). Traditionally, horses have been used in the production of antisera for the treatment of various diseases. For example, antisera against diphtheria, tetanus and snake venom are in high demand, particularly in places where vaccination programs are not available (Coghill et al., 1940; Hanly et al., 1995; Wilde et al., 1996; Wagner et al., 2009). In antibody production, domestic farm animals are used when large volumes of antisera and antibody are needed, or when smaller animals are not phylogenetically different enough from the antigen of interest (Coghill et al., 1940; Wagner et al., 2009). Additionally, with the advent of recombinant single-domain antibodies (termed nanobodies), which are now routinely used in both research and clinical applications, interest in using camelid species for antibody production has climbed sharply (Hamers-Casterman et al., 1993; Harmsen and De Haard, 2007; Muyldermans, 2013; Fridy et al., 2014; Klarenbeek et al., 2015). While large domestic animals are crucial for these reasons, their use is often prohibitive due to the high cost associated with using them, the facilities necessary to house them, and the ethical issues associated with experimentation on large mammals (Hanly et al., 1995; Wilde et al., 1996; Hein and Griebel, 2003; Conti et al., 2014).

Animals that fail to respond to antigen are a well-known problem during polyclonal antibody production (Garvey et al., 1977; Hanly et al., 1995). This is so common an occurrence that investigators will usually inject more animals than necessary to ensure an immune response. In smaller animals such as rabbits and mice this may not pose many issues, as the cost of upkeep for these animals and the amount of antigen required to inoculate them is low. However, large animals have high husbandry costs, and can sometimes demand large amounts of antigen to produce a strong immune response, so a large animal non-responder represents a substantial monetary setback that many researchers cannot afford. A method that can predict which animals will be likely non-responders to an antigen before it is purchased and set aside for biomedical research would thus be of significant utility. By reducing the chances of getting poor responding animals, time and money can be saved, and superfluous animal use can be avoided.

In humans, most studies of immune response have focused on person-to-person variation in response to single vaccines (Ovsyannikova et al., 2006; Poland et al., 2013; Tsang et al., 2014). However, it would be more beneficial if it were possible to predict what the response to one antigen in a single animal would be by looking at a previous response to a different antigen entirely. In one study on pigs, animals were bred to have either a high immune response or low immune response. One metric used to determine the strength of immune response was serum

* Corresponding author.
E-mail address: rout@rockefeller.edu (M.P. Rout).

http://dx.doi.org/10.1016/j.jim.2016.01.006
0022-1759/

concentration and avidity of anti-hen egg-white lysozyme antibodies (Appleyard et al., 1992; Mallard et al., 1992). When vaccinated against *Actinobacillus pleuropneumoniae*, pigs bred to have high immune responses had higher antibody titers and produced antibodies with higher avidity than those bred for low immune responses (Magnusson et al., 1997). This correlation implies that it may be possible to predict how well an animal will produce antibodies to one antigen based on their reactions to other antigens.

A convenient method we found to test a large animal's immune response is based on common vaccinations. The CDC suggests that all herd animals, especially animals that come in contact with humans and those that are of high value, be vaccinated against rabies (National Association of State Public Health Veterinarians, 2011). Additionally, *Clostridium perfringens* toxoids type B and type D and *Clostridium tetani* toxin are a cause of high morbidity and mortality in domestic farm animals, and prophylaxis via vaccination is a common herd management practice (Stiles et al., 2013). Because these immunizations are so prevalent, most large animals that will be used for antibody production will be vaccinated against these, or similar, antigens. Within a single farm, it is typical for a herd to be managed in a uniform fashion, removing many variables such as last vaccination date, vaccine brand, dose, and route of vaccination. Farms also tend to have standard adjuvants and injection protocols for experimental antibody production. All of these factors make it possible to readily analyze the response against vaccine antigens, and use those responses to predict how an animal will respond to future antigens.

During the course of previous work we observed that certain animals consistently produced high concentrations of antigen-specific serum antibodies to several highly divergent antigens. We hypothesized that if an animal is consistent with its level of antibody production, then future antigen response can be predicted by previous outcomes within a single animal. For this study we developed an ELISA method to analyze an animal's antibody titers against two common vaccinations, CDT and rabies, as well as mCherry and GFP antigens. Using this assay, we identified a correlation between antibody titers to common vaccines and titers to experimental antigens injected for the purpose of antibody production.

2. Materials and methods

The ELISA assay was designed based on standard published protocols (Bishop et al., 1984; Miura et al., 2008). Briefly, llama serum was saved from previous studies (Fridy et al., 2014); upon delivery, all serum was stored in 0.02% sodium azide to prevent microbial growth. Biographical and handling information for llamas and alpacas can be found in Tables 1 and 2. Llamas (*Lama glama*) and alpacas (*Vicugna pacos*) were used in previous and ongoing nanobody production efforts (Fridy et al., 2014). All animals were obtained from Capralogics, Inc., and all animal procedures were performed by Capralogics according to their Institutional Animal Care and Use Committee. Nunc-Immuno 96 well plates (Sigma) were coated with antigen. Vaccines were prepared for use as coating antigen as followed: Nobivac 3-Rabies Rabies vaccine (Merck) was diluted 1:16 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ pH 9.6). Vision CDT vaccine (Merck) was diluted 1:256 in coating buffer. To improve sensitivity and reliability of the assay, GFP and mCherry were crosslinked before coating. Proteins were prepared for use as coating antigen as follows: 50 μL of 4.3 mg/ml GFP or mCherry in PBS was mixed with 50 μL of 0.43 mg/mL BSA in PBS. 40 μL of ice cold 72% TCA was added and the mixture was incubated on ice for 10 min, then harvested by centrifugation at 14,000 rpm at 4 °C for 10 min. The pellet was gently washed with 500 μL of PBS and centrifuged again for 5 min. It was then resuspended in 200 μL 1% formaldehyde in PBS and sonicated, then incubated at room temperature for 1 h. GFP was then diluted 1:25 in coating buffer, and mCherry was diluted 1:500 in coating buffer. 100 μL/well for each antigen was plated (for final antigen amounts of 4 μg or 0.2 μg per well respectively) and plates were incubated overnight at 4 °C.

The next day coating solution was removed and plates were blocked for two hours at room temperature with 300 μL/well of 5% fat free skim milk in TBS. Plates were then washed three times with 300 μL/well wash buffer (0.1% Tween-20 in TBS). Serum bleeds diluted in dilution buffer (0.1% BSA, 0.05% Tween-20 in TBS) were used as primary antibody. In the case of animals 3484, 3485, 4761, and 4762, serum used for ELISAs was obtained after all boosters were given. For animals 5094–5098, serum for all antigens was obtained after two boosters were given. Serum was serially diluted 1:3 from 1:5000 to 1:10,935,000 for all antigens. 100 μL/well of primary antibody was pipetted onto plates, and plates were incubated for 2 h at room temperature. After the incubation, plates were washed three times with 300 μL/well wash buffer. Next, 100 μL/well 1:25,000 HRP-conjugated Goat α-Llama antibody (Bethyl) diluted in dilution buffer was used as secondary. Plates were incubated two hours at room temperature, then washed three times with 300 μL/well wash buffer. 100 μL per well of 3,3′,5,5′-Tetramethylbenzidine (TMB) (Thermo) was added and allowed to develop for 5 min, at which time the reaction was quenched with 100 μL 2 M H_2SO_4. Absorbance at 450 nm was measured with a BioTek Synergy NEO plate reader. Each experiment was performed in triplicate.

The absorbance at 450 nm was plotted against the log(concentration) of the reciprocal dilution of the serum used as the primary antibody. Titer cutoffs corresponding to a range of ODs were calculated in the following manner: A minimum OD and a maximum OD were chosen for each group of animals, and the titer values for five evenly spaced ODs in this range were interpolated from fitting a sigmoidal 4 Parameter Logistic curve to the data. The extreme values were selected by taking, e.g., the maximum OD for which a value was obtained for each animal and then finding the minimum of these values, ensuring that the selected cutoff would be in

Table 1
Biographical information for immunized llamas and alpacas.

ID Number	Species	Name	Sex	Age	Last rabies	Last CDT
3484	Llama	Barbie	Female	~8 years	N/A	N/A
3485	Llama	Pacino	Male	~7 years	N/A	N/A
4761[a]	Llama	Marley	Male	3 years	6/26/2013	3/26/2014
4762	Llama	Big Papa	Male	7 years	6/26/2013	3/4/2014
5094[a]	Llama	Marley	Male	4 years	6/26/2013	3/26/2014
5095	Llama	Briffit	Male	10 years	3/21/2014	3/21/2014
5096	Llama	Makeba	Female	8 years	3/21/2014	3/21/2014
5097	Alpaca	Ziti	Female	4 years	8/9/2013	3/26/2014
5098	Alpaca	Paulie	Male	2 years	1/31/2014	3/26/2014

[a] These numbers represent the same animal used in two different experiments.

Table 2
Animal immunizations. All initial immunizations were performed subcutaneously with complete Freund's adjuvant (CFA). All boosters were performed subcutaneously 21 days apart with incomplete Freund's adjuvant (IFA), except where otherwise noted. Preimmune bleeds were taken before initial injection. Test bleeds were taken 10 days after boosters; production bleeds were taken 10 days after the final booster. Animals in this chart are separated by species, antigens injected and project start date. Antigens in bold are those whose response was measured with ELISAs in this paper.

ID number	Antigen	First injection	Boosters	Project start date
3484	**GFP**-His_6	5 mg	5 mg × 5[b]	2/26/2010
3485	**GFP**-His_6	5 mg	5 mg × 5[b]	2/26/2010
4761[a]	$FLAG_3$-**mCherry**-$FLAG_3$	5 mg	5 mg × 3	5/17/2013
4762	$FLAG_3$-**mCherry**-$FLAG_3$	5 mg	5 mg × 3	5/17/2013
5094[a]	**GFP**	1 mg	1 mg × 3	4/25/2014
5095	**GFP**	1 mg	1 mg × 3	4/25/2014
5096	**GFP**	1 mg	1 mg × 3	4/25/2014
5097	**GFP**	1 mg	1 mg × 3	4/25/2014
5098	**GFP**	1 mg	1 mg × 3	4/25/2014

[a] These numbers represent the same animal used in two different experiments.
[b] The first two boosters were given 21 days apart, the following two were given 5 months later, 21 days apart from one another. A final booster was given 6 months after this, using complete Freund's adjuvant.

Please cite this article as: Thompson, M.K., et al., Optimizing selection of large animals for antibody production by screening immune response to standard vaccines, J. Immunol. Methods (2016), http://dx.doi.org/10.1016/j.jim.2016.01.006