

## Broad Neutralizing Antibodies to HIV Env and Other Complex Viral Antigens from Vaccinated Cows

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### Abstract

The idea of using antibodies to restrain HIV viral replication has been a growing interest since many years ago. HIV-patient serum with elite virus-neutralizing breadth has led to the preparation of broadly neutralizing antibodies (BrNAbs) with long and highly mutated CDRH3 domains that can neutralize a broad array of viral strains and prevent transmission in animal models. Recent advances have resulted in the discovery of BrNAbs that are more potent and can neutralize many HIV-1 subtypes. However, elicitation of these antibodies in infected individuals usually requires a long time of antigen exposure. Though BrNAbs are shown to be successful either therapeutically or prophylactically against HIV-1, production of these antibodies in bulk, commercial-sized batches are expensive and non-affordable particularly for poor countries. Therefore, more durable preventative or therapeutic strategies are required. Immunization of the cows with HIV Env is shown to be capable of producing 20 kg purified anti-HIV-1 BrNAbs and this amount could be sufficient for 2 million × 10 mg doses for formulation and pre-clinical testing as an HIV microbicide. In addition, bovine immunoglobulins typically have variable third heavy complementarity determining regions (CDRH3) that may potentially facilitate access to antigenic epitopes that are very difficult for other species to engage. Thus, cows could be engaged to elicit anti-HIV antibodies with the features of human BrNAbs and bovine colostrum could be a promising and cheap resource for the development of combination microbicides.

**Keywords:** HIV Env; Immunoglobulins; BrNAbs

### Introduction

A key component of a vaccine against human immunodeficiency infection (HIV) will be the production of BrNAbs capable of blocking infectivity of a diverse array of HIV strains. BrNAbs can emerge in some HIV-infected individuals after several years of infection [1]. The serum immunoglobulin G (IgG) from these “elite neutralizer” patients can neutralize many different HIV-1 strains across various different subtypes [2]. While waiting for the development of a fully effective HIV vaccine capable of eliciting these BrNAbs that are considered essential for blocking HIV transmission, other prevention strategies have turned towards testing passive infusions of broadly HIV-neutralizing monoclonal antibodies (BrNmAb) [3-6] derived from HIV-1 patients. These BrNmAbs have proven beneficial in long-acting therapeutic strategies, and they are now under evaluation as a systemic infusion with the BrNmAb to test their potential as a long-acting therapeutic [7,8] and also for transmission prevention [9-11]. The Antibody Mediated Prevention (AMP) trial will be considered as an alternative or adjunct to pre-exposure prophylaxis (PrEP) with antiretroviral drug combinations, such as Truvada [12]. The PrEP regimens have already demonstrated robust prevention against HIV transmission in some human trials in men who have sex with men (MSM). Effective PrEP requires high adherence in MSM [13]. However, PrEP has significantly lower efficacy in women, and requires long-term adherent dosing before any protective efficacy is achieved [14].

In spite of three decades of intensive effort, no prophylactic HIV vaccine has demonstrated strong protection from virus transmission.

The most challenging factor towards finding a compelling vaccine is finding a way to stimulate strong BrNAbs for the wide array of circulating viral strains. To date, vaccination against HIV-1 in human and animal models have yielded no or just narrowly focussed neutralizing antibodies, respectively. However, recent immunization studies in llamas and cows have resulted in induction of BrNAbs. Induction of BrNAbs in these animals is highly informative, as the studies from past 2 decades revealed that the antibodies from these species have unique structure facilitating antibody-antigen interaction through the less accessible epitopes [15,16]. The cows, particularly, produce exceptionally long CDRH3 which could be potentially advantageous in vaccine research developments. But furthermore, antibodies from Cow and Llama have production properties that illuminate potentially cheaper alternative approaches for passive antibody-mediated prevention than the extremely expensive approach pursued using humanized mAbs in the AMP study.

### Bovine colostrum as viral therapeutics

Immune modulation plays the most essential role in the recuperation of a patient from a life-threatening viral infection. An individual's immune system should act ideally to restrict and control the infection. The natural health benefit of bovine colostrum use has been known for centuries [17]. Bovine (cow and buffalo) IgG does not cross the placenta and IgG levels mount up in colostrum at levels 100-fold to 1,000-fold larger than in human colostrum. New born calves must feed on colostrum for continuing health and viability. Even human children can use cow or buffalo colostrum to attain health benefits [18,19]. Bovine colostrum contains 3 major groups of

components: nutritional components [20], immune factors [21] and growth factors [22].

The antiviral activity of colostrum is due to the presence of antibodies, lactoferrin, lactoperoxidase, lysozyme and other immune factors. These components control the pathogens by destroying their cell membranes or blocking the binding sites on the intestinal wall [19,23]. The cows can be vaccinated against a specific disease pathogen, which results in the production of antigen-specific antibodies and secretion in colostrum and milk. The immunization of the cow against a specific antigen may increase the specific antibody titer to 100-400 times in colostrum or milk, making a product known as hyperimmune colostrum. The total concentration of immunoglobulins in colostrum is 20-200 g/l (average 60 g/l). Among all classes, IgG1 with almost 75% is the predominant class in colostrum whey proteins followed by IgM, IgA, and IgG2, respectively [24]. One promising application of bovine colostrum and milk IgG is to provide passive immunity against diseases in other species, especially in humans. For instance, it has been suggested that consumption of immune milk from vaccinated cows is a potential means to control outbreaks of avian influenza, SARS, and other human respiratory diseases [25]. There are several studies evaluating passive immune protection via development and use of immune milk products [26-31].

Hyperimmune bovine colostrum is in particular helpful against human rotavirus [32]. Casein, a glycosylated protein, binds to the viral antigens directly using the glycosylated residue [33]. Vaccination of cows is a natural source of antibody production. However, the produced amount is not sufficient to cover the global requirement to reduce the number of death due to rotavirus-induced diarrhea [34]. Engineered *Lactobacillus rhamnose* GG with surface expressing the IgG-binding domains of protein G (GB1, GB2, and GB3) can bind to colostrums IgGs and enhance their potency in targeting rotavirus [34].

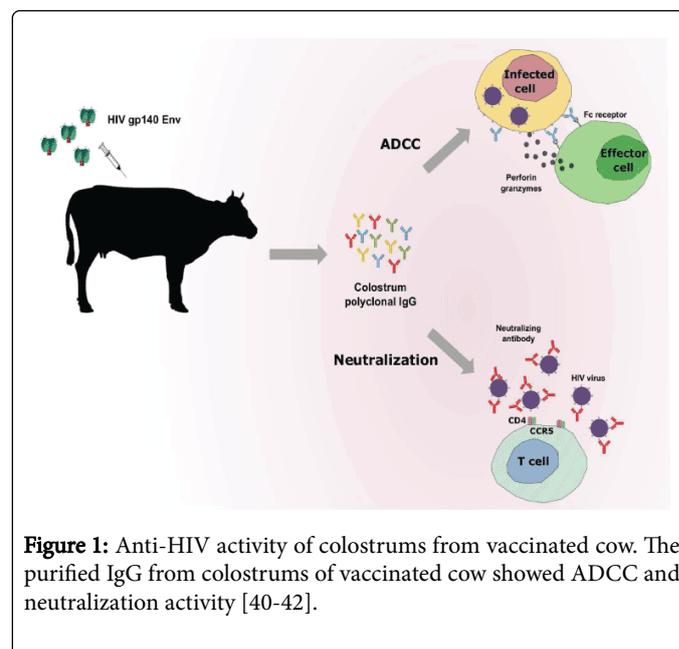
Furthermore, oral administration bovine colostrum to C57BL/6 mice improved immunity against influenza A virus (H1N1) through increasing natural killer cell cytotoxicity. One hypothesis is that colostrum components interact with innate receptors in the intestinal epithelium and stimulate these cells [35]. In another study, bovine colostrum IgG from a vaccinated cow with A/Puerto Rico/8/34 (PR8) influenza virus weakened the influenza symptoms in pre-treated BALB/c mice [36].

### Anti- HIV/AIDS activity of bovine colostrum

It was demonstrated that the infection-induced inflammation that occurs in the digestive tract of HIV-infected individuals can be reversed with bovine colostrum [37]. The bovine colostrum could possibly improve the tissue repair, mucosal integrity and also have direct antimicrobial actions [38]. Diarrhea is a common problem in AIDS patients, causing discomfort and malnutrition. Healthy people usually do not experience this complication. Human milk is effective in increasing circulating and tissue-resident helper T cells, consequently improving the immune system [39]. Bovine colostrum may be beneficial in HIV-infected individuals to re-establish the immune system and control the loss of T helper cells. It can also activate good health in the gastrointestinal immune system and promote mucosal integrity.

Elevated anti-HIV potency can be obtained from hyperimmune colostrum when bovine antibodies are directed against the HIV Envelope glycoproteins. Kramski et al. showed that polyclonal colostrum IgG fractions from cows hyperimmunized with HIV Env

gp140 demonstrate potent and broad HIV neutralization. In these studies, cows were immunized with HIV envelope (Env) gp140 for very long 10-month duration, before and after conception. The results showed that the cows were capable of producing broad cross-subtype strain neutralizing activity that was transferred at high titer ( $1:1 \times 10^6$ ) into colostrum. These antibodies include specificities that bind the highly conserved CD4bs on HIV Env trimmers and compete with the VRC01 mAb selected for analysis in the AMP trial and related BrNABs, like b12 [40]. Heydarchi et al. showed that bovine antibodies from vaccinated cow, not only bind to CD4bs but also can neutralize HIV infectivity through this region [41]. In addition, they showed that vaccination of the cows with oligomeric AD8 gp140 could induce the antibody response against the SOS-IP gp140 Env, which is the antigen with the closest structure to the functional Env protein [41]. In another study, IgG from hyper immune bovine colostrum from Env gp140 trimer vaccinated cows showed anti-HIV *Antibody-Dependent Cellular Cytotoxicity* (ADCC) activity. Bovine IgG could bind to Fc $\gamma$ -receptors (Fc $\gamma$ R) on human neutrophils, monocytes, and NK cells. Though anti-HIV-1 colostrum IgG displayed antibody-dependent killing, no killing was detected for non-immune colostrum IgG. ADCC activity was not seen with F(ab')<sub>2</sub> fragments and was only dependent on Fc and Fc $\gamma$ R [42] (Figure 1). Antibodies supporting ADCC activity correlated with protection in the RV144 trial, the only human vaccine trial to date that demonstrates any protective efficacy [43].



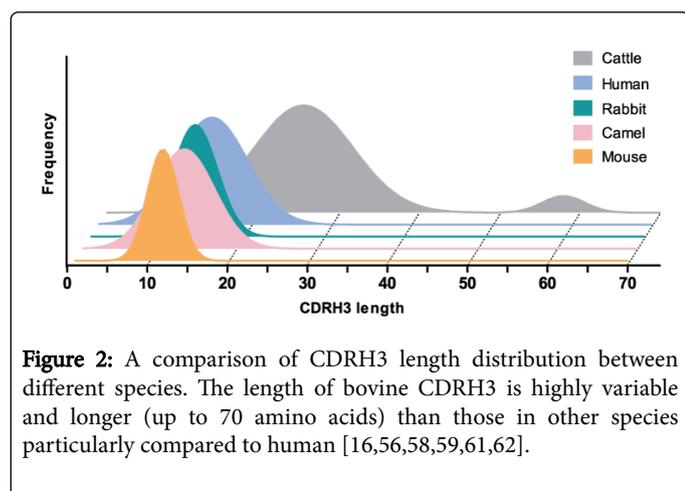
**Figure 1:** Anti-HIV activity of colostrums from vaccinated cow. The purified IgG from colostrums of vaccinated cow showed ADCC and neutralization activity [40-42].

### Bovine CDRH3 length goes beyond expectation

Potent antibody binding to key pathogen infectivity determinants requires a high level of evolution of the immunoglobulin CDRH3 domain to achieve high affinity for antigen [44]. The CDRH3 domain has the highest amino acid variability in IgG and plays the most critical role in the antigen binding interaction. This arises through two steps, first through a diversity generating mechanism using DNA rearrangement of variable (V), diversity (D) and joining (J) genes to create CDRH3 with novel gene sequences [45,46]. In the second step, somatic hyper mutation (SHM) drives further maturation to higher affinity and this occurs in the presence of antigen in the germinal

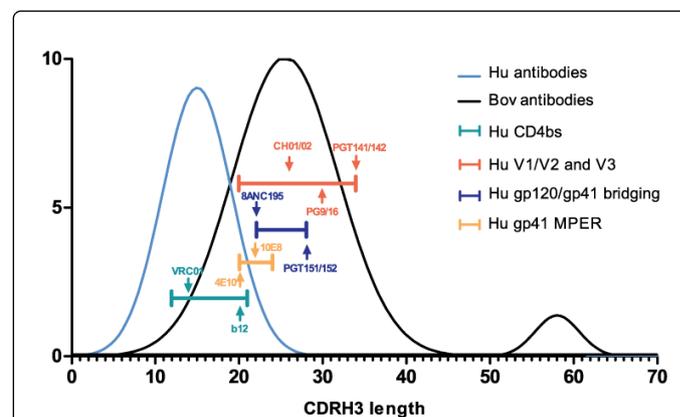
center of lymph nodes. In addition to VDJ recombination and SHM, the different use of D reading frames and variation in junction sites (P-nucleotides and addition of N-nucleotides) contribute to CDRH3 diversity [47,48]. The ultimate result is an affinity maturation of the variable region, especially the CDRH3 domain, and this increases the IgG neutralizing activity against HIV Env antigen [49].

The human CDR H3 length is normally 8-16 amino acids which contribute to form a flat or undulating binding surface along with the other heavy and light CDRs. Broad HIV-strain neutralizing antibodies obtained from long-term infected patients frequently contain an unusually long protruding CDRH3 [50-55]. The average CDRH3 size in mouse is 3 amino acids shorter than those in human [56]. Rabbit antibodies, on the other hand, present a length distribution similar to humans, with a slightly shorter CDRH3 (up to 23 residues) but keeping the highest frequencies around 13 residues [57,58]. In mice and rabbit, there is a bias against long CDRH3 antibodies development [60,61] which causes difficulties in using small animals for HIV vaccination trials [60]. Camelids present CDRH3 regions of between 3 to 24 residues, with the highest frequency between 13 and 17 residues [61,62]. In addition to heterogenic antibodies, camelids also present homogenous, light-chain depleted antibodies [63]. These antibodies, called heavy chain antibodies, have a variable region called VHH, which has its own CDR3 region. A comparison of VHH with conventional antibodies shows that the former group presents larger CDH3 length (between 7 and 24 residues) than the VH of conventional antibodies [61]. Heavy chain camelid antibodies offer the potential for high-level production in conventional bacterial and yeast biofermentation which may lead to large-scale, relatively low-cost production [64]. In contrast, the length of bovine CDRH3 is highly variable and longer (up to 70 amino acids) than those in other species particularly compared to human [16,65-68]. The antibodies with ultralong CDRH3 can comprise up to 10-15% of the entire bovine IgG repertoire (Figure 2) [16]. Crystal structure of the bovine antibodies with ultra-long CDRH3 shows that these long variable domains contribute to an unusual structure. These CDRH3 regions join to a particular set of lambda light chains with limited diversity [67].



In the naïve B cell pool, 3.5% of B cells have CDRH3  $\geq$  24 residues and 0.43% of them contain very long CDRH3  $\geq$  28 residues [69]. However, this percentage is substantial with consideration of the total potential number of 1012 different antibodies in the human B cell repertoire. This means that B cells with long CDRH3 can actively contribute in the human humoral immune system. It appears that B

cells producing antibodies with long CDRH3 are selected with HIV-1 Env to generate BrNAbs targeting deep epitopes of HIV-1 Env. Furthermore, there is a meaningful relationship between long CDR H3 (20-34 residues) and BrNAbs especially in antibodies targeting the glycan-related V1/V2 and V3 category, the gp120/gp41 bridging region category and the gp41-MPER category. This contrasts with the average 16 residues of CDRH3 in the antibodies elicited by other viral antigens [54,70]. The role of such long CDRH3 (18 residues) in b12, as a member of CD4 binding site antibodies, is to access a glycosylation site [71,72]. However, the CDRH3 in CD4bs BrNAbs are shorter than the glycan-related V1/V2 and V3 category. The longest CDRH3 belongs to PG9-like and PGT128-like BrNAbs that forms a sub-domain that is important for neutralization (Figure 3). These CDRH3 regions help the antibodies to penetrate the glycan shield of Env trimer then interact with the V1/V2 and/or V3 region of gp120 [73]. Also, CDRH3 is an important component in gp41-reactive antibodies such as 2F5 (CDRH3 of 22 residues) and 4E10 (CDRH3 of 18 residues) which performs the additional activity causing a hydrophobic interaction with the membrane [74-80] and forming a loop to contact the highly conserved hydrophobic residues on gp41 [52,81-83].



**Figure 3:** Comparison of bovine CDRH3 length with the CDRH3 size of human BrNAbs. The bovine CDRH3 covers the size of CDRH3 in all human BrNAbs. Bov: bovine; Hu: Human antibodies [16,50-55].

### Aromatic residues in bovine CDRH3 pave the way toward HIV neutralization

Previous studies on human immunoglobulins derived from HIV patients illustrate that although the presence of Cys and aromatic residues is a rare feature, these amino acids play a crucial role for HIV BrNAbs in epitope binding or virus neutralization. The substitution of aromatic residues, mainly Trp or tyrosine (Tyr), in MPER binding antibodies reduces the neutralization activity of these antibodies [74,84]. Also, the importance of Cys and aromatic residues such as Trp is shown in neutralization activity of CD4bs BrNAbs antibodies [85,86], highlighting the significant function of these residues in either directly in the antigen binding interaction or involvement in shaping the required structure for epitope binding.

The activity of patient-derived HIV-1 BrNAbs requires an extensive affinity maturation in the lymph node germinal centers in comparison with other human IgG antibodies against most other pathogens, or with poorly neutralizing HIV antibodies [70,87,88]. The most

important of the maturation mechanisms is SHM particularly in the heavy variable region [89-95]. A high degree of amino acid SHMs ranging from 9.4% for M66.6 (gp41 MPER antibody) up to 47.9% for VRC06 (CD4bs antibody) is a characteristic feature of HIV-1 BrNABs [96]. The importance of SHM is demonstrated by low or absent reactivity of predicted un-mutated germline ancestors of BrNABs [97,98].

In *Bos taurus*, the *V* gene is almost limited to one family (gl.110.20) [65,66,68]. However, the bovine immune system has a robust function which implies that some diversification mechanisms are employed to help the immunoglobulins to target epitopes of diverse pathogens. High level SHM in the immunoglobulins is one of the main strategies that bovine immune system uses to provide enough antibody evolution and adaptation for efficient antigen recognition [99]. There are other major factors that result in the bovine immunoglobulins being exceptional amongst immunoglobulins derived from other species. Most unique is that bovine IgG can have ultra-long CDRH3 that form a  $\beta$  strand “stalk” that supports a structurally classified, disulfide-bonded “knob” domain. To create diverse antigen binding surfaces, a Cys diversification is employed through a single V(D)J event in cows which reshape the knob domain in the ultra-long CDRH3 regions [16]. In bovine, CDRH3 domains are particularly elongated, and Cys residues are normally frequent forming structures that involve disulfide bonds [67]. The DH2 germline gene encodes repeating motif of Gly-Tyr-Gly which could be mutated into cysteine [16,66]. In another study, the critical contribution of aromatic residues (particularly Trp and His) for HIV Env binding has been revealed (Heydarchi et al. submitted). The long CDRH3, high level of SHMs and presence of Cys and aromatic residues are parts of the normal bovine immunoglobulin that are comparable to what was observed from human neutralizing immunoglobulins which in contrast, require a long time to develop and are not observed in normal antibodies [1].

## Limitation and Challenges

There are some challenges with the production of virus-specific bovine antibodies from colostrum. Our experience with HIV Env antibodies suggests potent neutralizing IgG antibodies required a relatively long maturation time when produced by pregnant cows [40]. Furthermore, the large body mass of the cows may require much more vaccine compared to other animals [40,100-102]. Additionally, highest concentrations of colostrum antibody are found in the first colostrum and this must be collected immediately after the unpredictable event of calving. Nevertheless, production of large scale anti-viral components and antibodies compensates for this drawback.

Other potential issues may arise when animal-derived un-purified polyclonal antibodies from colostrum are used for clinical applications and these materials would usually require purification of IgGs [103]. In general, quantitative and qualitative batch-to-batch variation is the another potential obstacle with the use of polyclonal antibodies from animals [104]. Though passive immunization with the bovine polyclonal antibodies can prevent or control infection [26-31,40], a general problem for polyclonal antibodies is that a large portion of stimulated antibodies will target non-neutralizing epitopes and titer of specific antibodies may be low [42]. However, one strategy to cope this problem may be the production of the monoclonal BrNABs targeting conserved epitopes among different subtypes of a virus. Finally humanization of the bovine monoclonal antibodies will be required to modify the protein sequence towards antibodies naturally tolerated in humans [104,106] therefore reducing anti-antibody responses [107].

On the whole, though there are some challenges existing with the use of bovine polyclonal antibodies from colostrum, it seems that the advantages of repertoire diversity and production scale outweigh the disadvantages when bovine-derived antibodies are employed in the control and/or treatment of antigenically complex viruses, such as HIV.

## Conclusion

The colostrum-derived IgG could improve the immunity against different viruses including HIV. It is curious that bovine yields remarkable high titers of BrNABs after vaccination but that this does not occur following vaccination of other species. Vaccinations with HIV Env gp140 trimmers that have evolved to evade human antibody immunity may present epitopes in bovine that are anergic in primates [108]. Furthermore, as previously shown the pre-existing immunity against human microbiota could mislead the immune system to induce non-neutralizing antibodies [109]. So, it is also possible that the microbiota that shapes the polyfunctional B cell precursors that spawn BrNABs in humans are different to those in a ruminant, like the bovine, leading to a more rapid and reproducible production of neutralizing antibodies. Finally, the unprecedented long length of the CDRH3 regions of the bovine IgG [65,66] leads to V-regions that make long CDRH3 with an average CDRH3 length that is equal to the maximum size for human CDRH3 frequently observed in BrNAB from human [54,71]. In addition, high a rate of SHM is used in the bovine antibodies [109] may lead to elicit BrNABs with characteristic highly mutated. Indeed, the bovine antibodies from vaccinated cows could eventually benefit HIV-infected individuals by means of their extraordinary features.

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