



Resolution of an immunodiagnostic dilemma: Heavy chain chimeric antibodies for species in which plasmacytomas are unknown

J.E. Butler^{a,d,*}, N. Wertz^{a,d}, X.-Z. Sun^{a,d}, J.K. Lunney^{c,d,1}, S. Muyldermans^{b,d}

^a Department of Microbiology and Interdisciplinary Immunology Program, University of Iowa, Iowa City, IA 52242, United States

^b Animal Parasite Disease Laboratory, ANRI USDA ARS, Beltsville, MD, United States

^c Laboratory of Cellular and Molecular Immunology, Vrije Universiteit Brussels, Pleinlaan 2, 1050 Brussels, Belgium

^d Department of Structural Biology, VIB, Brussels, Belgium

ARTICLE INFO

Article history:

Received 5 June 2012

Received in revised form 10 July 2012

Accepted 21 July 2012

Keywords:

IgG subclasses

Allotypes

Swine

In vitro synthesis

ABSTRACT

The immunoglobulin (Ig) genes of many vertebrates have been characterized but IgG subclasses, IgD and IgE proteins are only available for three species in which plasmacytomas occur. This creates a major problem in the production and specificity verification of diagnostic anti-Ig reagents for the vast majority of mammals. We describe a novel solution using the swine system with its eleven different variants of IgG. It involves the *in vitro* synthesis of chimeric porcine-camelid heavy chain antibodies (HCABs) that do not require light chains and therefore only a single transfection vector. The expressed chimeric HCABs are comprised of the camelid VHH domain encoding specificity for lysozyme and the hinge, CH2 and CH3 domains of the various porcine IgGs. These HCAB retain their antigenic integrity and their ability to recognize lysozyme. The engineered specificity assures that these HCAB can be immobilized in native configuration when used for testing the specificity of anti-swine IgG antibodies. Comparative data to illustrate the importance of this point are provided. These are now available for use in hybridoma selection and as reference standards for evaluating the specificity of currently available anti-swine IgG antibodies.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

In the field of immunology the ability to quantify immunoglobulin (Ig) levels and to identify which isotypes or subisotypes of antibodies are involved in a particular humoral immune response, depends on using specific anti-Ig reagents. This requires that the isotype of interest be biochemically purified and then be used to prepare specific monoclonal (mAb) or polyclonal antibodies (pAb). Testing the specificity of these antibodies and selecting suitable hybridomas requires that purified standards for each Ig be available in a native (non-denatured) configuration. Except for rodents and humans and for IgM, IgA and for some subclasses in ruminant artiodactyls, there are no purified IgG subclasses, IgD or IgE proteins available for ~90% of the most commonly studied mammals that can be used for hybridoma selection and as reference standards for testing isotype-specific reagents (Table 1). Anti-Ig reagents are

readily available for species in which: (a) Ig including IgG subclass proteins can be biochemically purified or (b) plasmacytomas occur or can be induced (Table 1). In rodents, these Igs can be purified from the ascites fluid of Pristane-induced plasmacytomas or naturally from the sera of humans with multiple myeloma (Potter and Boyce, 1962; Kunkel, 1963). Because plasmacytomas do not occur naturally in most mammals, or have not been successfully induced, their IgG subclasses, IgD and IgE are only known from gene sequences (Table 1). A separate problem exists for mammals such as insectivorous bats that now suffer population collapse due to white-nosed syndrome (Bleher et al., 2009). In these tiny bats and from other species in this category, the amount of serum that would be needed for physical chemical purification of any Ig would be in violation of animal use guidelines. However, Ig gene sequences are available for these and most all of the species listed in Table 1 (Bratsch et al., 2011; Butler et al., 2011).

IgG is the flagship Ig of mammals and is not present in any other vertebrate class. IgG comprises >80% of the serum Ig in mammals and has diversified into subclasses in most mammals with the number ranging from 1 to 7 (Table 1; Butler, 2006). It is recognized from studies in humans, cattle and lab rodents that there is a division of labor among IgG subclasses (Butler, 1983; Lefranc and Lefranc, 2001; Mossmann and Coffman, 1989; Ravetch and Kinet, 1991). This structure-function relationship among subclasses has

* Corresponding author at: Department of Microbiology and Interdisciplinary Immunology Program, University of Iowa, Iowa City, IA 52242, United States. Tel.: +1 319 335 7776.

E-mail addresses: a.balm@nki.nl, john-butler@uiowa.edu (J.E. Butler).

¹ Research supported by grant 58-1265-5-053 from the National Pork Board and UMASS-DVLP Subcontract 11-006340.

Table 1

Availability of purified immunoglobulin from common mammals.

Species	IgM	IgD	IgG ^a	IgE	IgA ^a	Purified polyconals	Purified paraproteins ^c
Mouse	1	1	4	1	1	IgM, IgA	All isotypes and subisotypes
Rat	1	1	4	1	1	IgM, IgA	All isotypes and subisotypes
Human	1	1	4	1	2	IgM, IgE, IgA ^b	All isotypes and subisotypes
Rabbit	1	0	1	1	13	IgM, IgG, IgA ^b	None
Bovine	1	1	3	1	1	IgM, IgG1, IgG2, IgA	None
Sheep	1	?	2+	1	1	IgM, IgG1, IgG2, IgA	None
Camel	1	?	5	?	?	Potentially ^d	None
Horse	1	1	7	1	1	IgM, IgA	None
Pig	1	1	6	1	1	IgM, IgA	None
Cat	1	?	2+	1	?	Potentially ^d	None
Dog	1	1	4?	1	1	Potentially ^d	None
Bat	1	1	4+	1	1	Potentially ^{d,e}	None
Opossum	1	?	1	1	1	Potentially ^d	None
Platypus	0	?	2	1	2	Potentially ^d	None

^a Number of expressed subclass proteins; pseudogenes are not included.^b Not individual subclasses of IgA.^c Purified from mouse and rat ascites or human sera from myeloma patients with IgM, IgA and IgG plasmacytomas.^d Theoretically possible for IgM, IgA and pan IgG.^e Only from large fruit bats.

lead to the view that measuring subclass immune responses can help to further understand the significance of a particular subclass in protecting or damaging the host. IgA subclasses also occur in mammals and in humans, these differ in susceptibility to proteolysis; human IgA1 is more susceptible than IgA2 to bacterial IgA proteases (Burnett et al., 1989; Kilian and Russell, 2004; Plaut et al., 1975). IgA subclass diversification is most extreme in rabbits; thirteen have been described. Rabbit IgA subclasses are differentially expressed among various lymphoid tissues suggesting they may also differ in function (Spieker-Polet et al., 1993). Allotypic variants of porcine IgA also occur but *Haemophilus proteases* cleave both allotypes (Brown et al., 1995; Mullens et al., 2011).

Diversification of IgG into subclasses appears to have occurred after speciation (Butler et al., 2009; Kehoe and Capra, 1974) so that same name subclasses in different mammals are not homologous proteins. This means that reagents generated to a particular IgG subclass, e.g. IgG1 in one species are rarely and unreliably cross-reactive to the same name subclass in other species. Therefore, phylogeny suggests that it is dangerous to extrapolate the function of e.g. IgG1 in one mammal to the same name IgG1 in other mammals (Butler et al., 2009). Thus there is a need for developing subclass-specific and/or even allotype-specific reagents for use in each species. Therefore the method we describe herein should be of broad appeal to investigators working with all species in which plasmacytomas do not occur, in which plasma volumes are tiny and even in mice and humans in which the specificity of mAbs or polyclonal reagents has been limited to a pool of variants available as paraproteins.

We chose the porcine system in which to test the technology because: (a) it offers a complex IgG systems, (b) focuses on a species of importance as vectors for human pandemic viral disease (Lorusso et al., 2012), (c) this species has been used in xenotransplantation (Auchincloss and Sachs, 1998), (d) isolator piglets are valuable for understanding the role of bacterial colonization and neonatal viral exposure (Butler et al., 2002; Butler and Sinkora, 2007; Lemke et al., 2004), (e) the swine is now a model for a major human disease e.g. CFTR knockout piglets as models for cystic fibrosis (Rogers et al., 2008) and (f) engineered transgenic pigs are being generated for eventual production of humanized antibodies (Mendicino et al., 2011; Ramsoondar et al., 2011). The methodology we describe is for the preparation and testing of diagnostic reagents and not intended to be a new method for developing chimeric antibodies for human therapy.

The antigenic heterogeneity of porcine IgG has been known for some time (Kaltreider and Johnson, 1972; Metzger and Fougereau,

1967; Prokesova and Rejnek, 1973; Rapacz and Hasler-Rapacz, 1982) but efforts to purify individual variants using ion-exchange chromatography and/or differential affinity for Protein A were unsuccessful (Dillender, 1988). Nevertheless, IgGs recovered from the extremes of the ion exchange spectrum were designated IgG1 and IgG2 (Metzger and Fougereau, 1967) in the same tradition used in the guinea pigs and ruminant artiodactyls (Benacerraf et al., 1963; Butler, 1969, 1983). Commercially available mAb designated as specific to the two groups, i.e. “subclasses”, are available. However the supplier has provided no evidence for the specificity of the mAbs for the six IgG subclasses of swine (Butler et al., 2009, 1994; Kloep et al., 2012). A major obstacle for such suppliers is the unavailability of purified forms of the various IgG subclasses and their allotypic variants. This problem is not restricted to swine but to any species for which IgG subclasses cannot be biochemically purified. While human myeloma proteins or IgG from mouse plasmacytoma are available, they are unlikely to provide the full range of allotypic variants. This immunodiagnostic problem is exacerbated because, unlike pharmaceuticals, there are no industry standards and no regulatory agency.

We describe a simple technology for generating Ig subclass and allotypic standards when only gene sequences are available. This technology involves the preparation of chimeric heavy chain antibodies with designed antibody specificity (HCABs). The camelid family of mammals is fairly unique among mammals in the regular production of HCABs (Hamers-Castermann et al., 1993) although this is also seen with IgNAR in the nurse shark (Greenberg et al., 1995). In camelids, these HCABs utilize a special group of VH gene segments during VDJ rearrangement known as VHH (Fig. 1; Muyldermans et al., 1994; Nguyen et al., 2000). These HCABs have affinities comparable to conventional IgG antibodies that have both heavy and light chains (De Genst et al., 2006). Since the binding specificity of these HCABs does not require light chains, *in vitro* transfection utilizes one simple vector generating a product that does not need to be assembled from heavy and light chain as is true for conventional Igs. The chimeric DNA constructs we describe contain a camelid VHH domain gene encoding specificity to hen's egg lysozyme (HEL) which is linked to the exons encoding the hinge, CH2 and CH3 domains of swine IgG. However, the constant region exons could be from any species which extends the appeal of the technology. These chimeric HCABs are especially desirable because they can be immobilized through their VHH-encoded binding site to HEL on microtiter wells so the specificity of any mAbs or pAbs can be tested against the IgG protein in native configuration. This avoids the potential problem of adsorption-induced denaturation

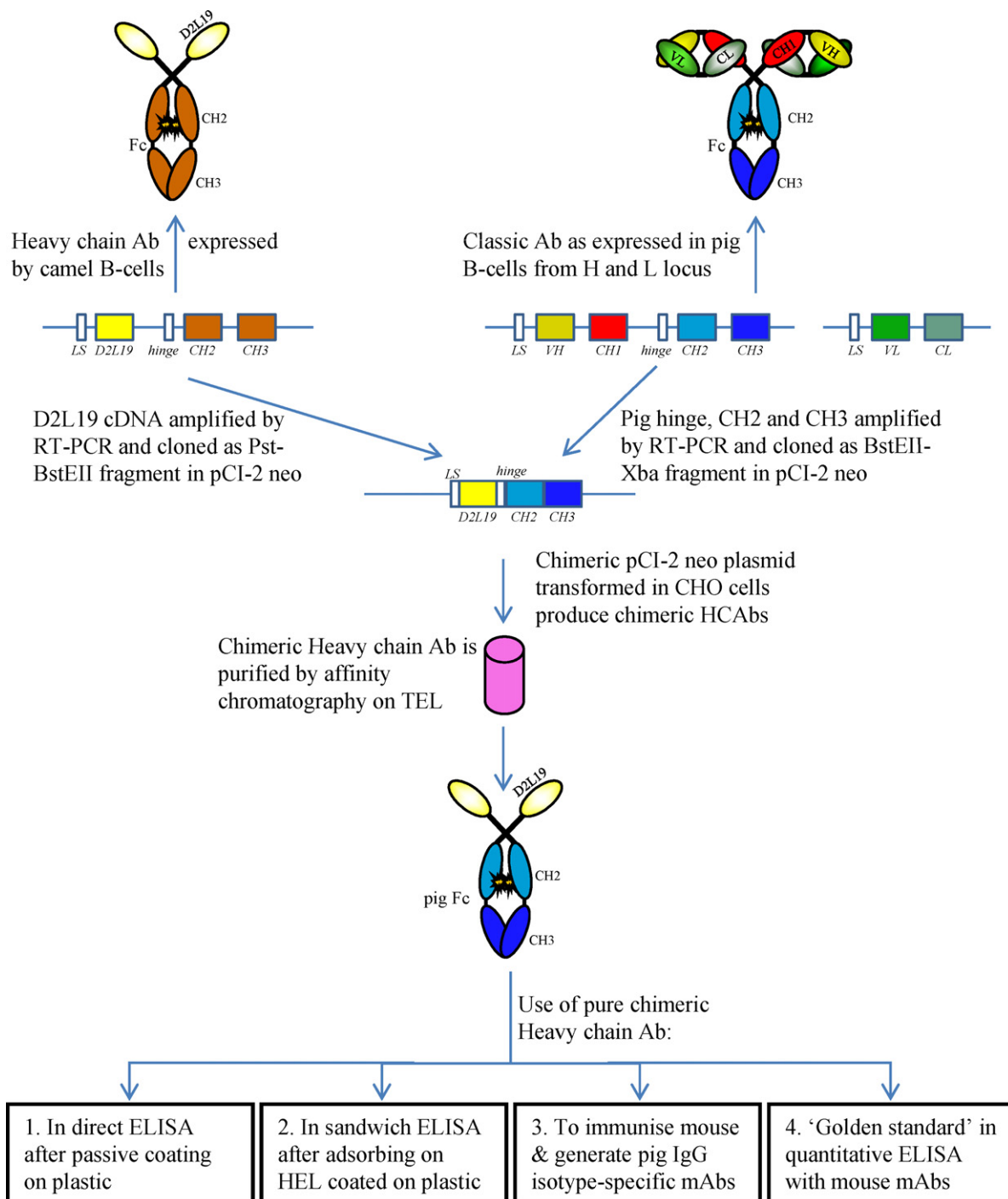


Fig. 1. Structure of heavy chain antibodies (HCAb) from the camelids that lack light chains *versus* the conventional mammalian IgG antibody. VHH is the variable heavy chain fragment that confers antigen specificity in the absence of light chains.

when proteins are directly adsorbed on a synthetic hydrophobic solid phase (Butler et al., 1993; Butler, 2000; Dierks et al., 1986). Furthermore, when these chimeric HCABs are immobilized by binding to HEL-coated wells, they are oriented in the same manner in which they would normally bind their antigens *in vivo*, i.e. with their Fc regions exposed.

This report details the construction of the transfection vector for porcine-camelid chimeric HCABs, the *in vitro* production of chimeric HCABs and their use in determining the specificity of available mAbs to porcine IgG. The methods described herein provide a means to obtain purified IgG subclass proteins and their allelic variants from any species in which the C γ genes have been cloned. This

technology makes available subclass proteins and thereby provides a solution to the problem of hybridoma selection and of testing the subclass specificity of mAbs.

2. Materials and methods

2.1. Source of the C γ gene cDNA

cDNA transcripts of porcine C γ genes were prepared as previously described (Butler and Wertz, 2006; Butler et al., 2009). Major differences in the deduced protein sequence are highlighted in Fig. 2. Fig. 2 also indicates (a) which IgG subclasses appear to

Major sequence differences, relative transcription among the porcine IgG variants and μ g available

IgG variant	IMGT	Critical Sequences			Relative Transcription		μ g available
		Hinge	Upper CH3	Lower CH3	Newborn	PIC	
IgG1 ^a	IGHG1-01	GTKTKPF---CPICPGCE-VA	TISKAIGQSREPO	LAVDKRARWDHGETFE	34.8 %	49.7 %	144
IgG1 ^b	IGHG1-02	.IHQPQT-----		DK..	30.8 %	22.9 %	1056
IgG2 ^a	IGHG2-01	A..-SP	I...K..T....	FS...S.QG.GI.Q	3.1 %	8.1 %	*
IgG2 ^b	IGHG2-02	A..-SP	I...K..T....	FS...S.QG.GI.Q	2.6 %	2.3 %	180
IgG3	IGHG3	DIEPPT.ICPEICS..AALVLG	T.P..V..	V..QR.DL.Q	22.8 %	5.0 %	140
IgG4 ^a	IGHG4-01	A..-GP	I...K..T....	FS...S.QR.D..Q	0.3 %	2.7 %	450
IgG4 ^b	IGHG4-02	.IHQPQT-----	I...K..T....	FS...S.QG.GI.Q			910
IgG5 ^a	IGHG5-01	-----RG-----	K.VV.S.E	LQS.GI-H	8.7 %	9.0 %	246
IgG5 ^b	IGHG5-02	.K...PR-----	K.VV.S.E	FS.E.F..HS.GI-H			189
IgG6 ^a	IGHG6-01	-----R-----	I...K..T....	FS...S.QG.GI.Q	0.1 %	0.5 %	216
IgG6 ^b	IGHG6-02	-----R-----	I...K..T....	FS...S.QR.DT.Q			480

Fig. 2. The sequence of the hinge region of the various porcine IgG subclasses and allotypic variants. Also given is the transcription frequency for these γ genes and the quantity currently available. * There is no change in the protein sequence from IgG2^b so there is no need to produce IgG2^a.

be most important based on transcript frequency and (b) the current availability of each HCAB. Regarding the potential importance to the host, IgG3 comprises 40–60% of γ transcripts in newborns whereas IgG1 comprises the major proportion in adult swine.

2.2. Construction of chimeric HCAB expression vector

A panel of anti-HEL nanobodies, i.e. antibodies comprised of only the VHH from camelids, was generated in the laboratory (De Genst et al., 2006). In initial studies, these were selected for high affinity for HEL and used to construct a modified pCI-2 vector. However *in vitro* generated chimeric HCABs could not be easily eluted from HEL affinity columns and were instead purified on protein A/G columns. Naturally, these preparations were contaminated with bovine IgG from the FCS in the culture media. Therefore we selected a second anti-HEL nanobody (D2L19) with cross-reactivity to turkey egg lysozyme (TEL) which allows recovery of the chimeric HCAB from culture supernatants on TEL affinity columns (see Section 2.4).

Fig. 3 shows the modified pCL vector (Invitrogen, Carlsbad CA; pc DNA3.1) containing the cAb-Lys3 nanobody sequence in fusion with the hinge-CH2-CH3 region of a swine γ gene. Creation of the desired construct required that cAb-Lys3 be replaced by nanobody D2L19. The final construct contains the leader sequence, nanobody D2L19 and the hinge-CH2-CH3 domains of swine IgG1^a followed by two stop codons.

2.3. Optimization of transfection of CHO cells

Optimal conditions were established using a series of transient transfections. These were tested in conventional (RPMI + 10% FCS, 1% penn/strep (CM)) and serum-free media; the latter to avoid contamination with bovine IgG from FCS. In these studies both Eugene HD (Roche) and PEI (polyethylenimine) were used. Eugene and the use of conventional media were selected since cells thrived better

and purification of chimeric HCAB could be done on TEL affinity columns (see Section 2.4). Two days after transfection, CHO cells were treated with G418 (1%) and suspensions of these cells were diluted and plated at one cell/well. After ten days in culture, supernatants were tested by ELISA for their ability to bind immobilized HEL (see Section 2.5) and the highest producing monoclonal selected, transferred to 24 well plates after 1 week, and retested by ELISA. We also measured the production of chimeric HCAB when multiple clones were present or when cultures exceeded 10 days. We found multiclonal and older cultures to be less productive.

2.4. In vitro production and purification of chimeric HCAB

High-producing monoclonal of transfected CHO cells were cultured in 55 ml of CM containing 1% G418 in multiple T-150 flasks until cell confluence, ~7–10 days. The supernatant was then passed over a TEL affinity column. Since TEL is not commercially available, it was purified from turkey egg whites using a sulfopropyl cation exchange column. The column was eluted with a salt gradient from 0.1 M NaCl in 20 mM Tris at pH 8.0 to the same buffer containing 1 M NaCl. The eluting fractions were monitored for lysozyme activity using 100 μ l of dried *Micrococcus lysodeikticus*, suspended in PBS in wells of 96-well plates, to which 10 μ l of each column fraction was added and lysis measured by OD at 450 nm. Fractions that were able to break down the bacterial cell wall were subjected to size exclusion chromatography on Sepharose S300 and the fraction eluted at a molecular weight of TEL (~15Kd) collected and re-tested for lysozyme activity.

TEL affinity columns were prepared using the AminoLink plus system (Pierce Chemicals, Rockford IL). Coupling was done at pH 7.2 in 0.1 M phosphate buffer containing 0.15 M NaCl. We found 90% coupling efficiency resulting in columns containing 7–9 mg of TEL. 300 ml of media were passed over these columns which resulted in the recovery of 1–2 mg of HCAB. Chimeric HCABs were eluted by acid shock using 0.025 M citrate buffer, pH 2.5, fractions neutralized using 1 M Tris, concentrated over a Amicon/Millipore PL membrane with a 10 Kd cut-off, the protein content determined by NanoDrop spectrophotometry and the product evaluated on SDS-PAGE (Fig. 4). Each sample was then treated with 10% glycerol to discourage proteolysis and to stabilize the dilute protein.

2.5. Detection of chimeric HCAB in culture fluid

NUNC MaxiSorp plates or removable wells were incubated for 3 h at 37 °C with 200 μ l of HEL or TEL (5 μ g/ml) in 0.1 M carbonate coupling buffer, pH 9.6 (Engvall and Perlmann, 1972). After incubation for 3 h at RT, plates were washed and 100 μ l of culture supernatant was added to 100 μ l of PBS-T previously added to the wells. After incubation at RT, bound HCAB was detected

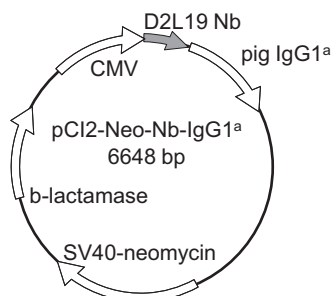


Fig. 3. The pCL2 plasmid containing the VHH nanobody specific for HEL and the site for inserting the hinge, CH2-CH3 gene sequences of the swine γ variants.

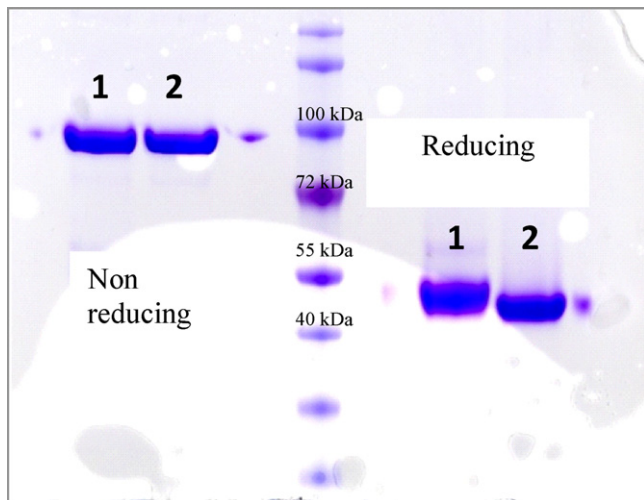


Fig. 4. Analysis of HCABs from the supernatant of clones 2Z10 (IgG1^a) and 4B11 (IgG3) using SDS-PAGE. Legend on the figure.

using biotinylated rabbit anti-swine IgG (B78; Butler, 2000) followed by Extra avidin-AP conjugate and a phosphatase substrate (PNP; Sigma–Aldrich, St Louis, MO). Results were read on a BioTech EL80 plate reader.

2.6. Polyacrylamide electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described by Laemmli, 1970 and as previously described for porcine and bovine immunoglobulins (Butler, 1983). Further details are provided with Fig. 4.

2.7. Test for antigenic integrity

HEL was adsorbed on NUNC MaxiSorp or Immunulon 2 microtiter wells in the manner described above. After incubation and washing, HCAB in PBS-T (PBS-T) were incubated overnight at RT on HEL-coated wells over a range from 1 to 10 µg/well. The plates were then washed and detection made using biotinylated rabbit anti-swine IgG, Extra avidin-AP and using PNP as the substrate as described above. In an assay conducted in parallel, the HCABs were adsorbed directly to microtiter wells in coupling buffer at 0.1–1 µg/well and after incubation and washing, the same detection system described was applied. These studies established that the addition of 1 µg/well of HCAB to HEL-coated plates or direct adsorption at 0.5 µg/well was saturating.

2.8. Specificity of mAbs to chimeric HCABs

HCAB were immobilized by affinity binding to HEL or directly adsorbed as described above. After immobilization the plates were washed and a dilution of mAbs sufficient to saturate the immobilized HCAB added in PBS-T. The dilution was established in preliminary tests by determining a dilution able to saturate the target; in most cases this was 1:200. The actual dilution is given in the figure legends and in Table 2. As a negative control we used anti-fluorescein mAb 4-4-20 (Table 2). After incubation overnight at RT, plates were washed and the bound HCAB detected using biotinylated goat anti-mouse IgG prepared in the laboratory that had been adsorbed on a swine IgG column to remove all cross-reactivity to swine IgG. Biotinylation were done using PEG-biotin (Pierce-Thermal, Rockford, IL) according to the instructions of the manufacturer. Mouse mAbs tested are described in Table 2.

Table 2
Anti-swine IgG and control reagents.

Designation	Source	Indicated specificity	Dilution tested
Monoclonal 34.1.1d	Bianchi	Porcine IgG1	1:20
Monoclonal 23.4931	Bianchi	Porcine IgG2	1:200
Monoclonal 3H7	Paul	Porcine IgG	1:100
Monoclonal 4-4-20	Voss	Flourescein hapten	1:200
Monoclonal M136	Conant Lab	Porcine IgG	1:200
Monoclonal M19	Conant Lab	Porcine IgG	1:200
Polyclonal B78	Butler Lab	Porcine IgG-Fc	1:500

3. Results

3.1. Recovery and characterization of HCABs

150–300 ml of culture supernatant was pumped through HEL affinity columns containing 7–9 mg of HEL. The HCAB was eluted as described in Section 2, neutralized, concentrated and the IgG concentration determined spectrophotometrically. The recovered product was then evaluated by SDS-PAGE under reducing and non-reducing conditions (Fig. 4). This generated a 100 Kd product which after reduction generated a 50 Kd product. This outcome is consistent with an HCAB comprised of two chimeric heavy chains covalently linked by disulfide bonds.

3.2. HCAB retain their antigenic properties when antigen-bound or adsorbed on plastics

Fig. 5 shows that polyclonal B78 detected all HCABs when immobilized either by binding to HEL or after direct adsorption on plastic. B78 showed no allotype bias but generally recognized HCAB bound to HEL less well. While not tested using ¹²⁵I-labeled HCAB, we suspect this reflects the fact that less HCAB is immobilized when bound to antigen than when directly adsorbed on plastic.

3.3. Anti-swine IgG mAb display subclass and conformational specificity

Figs. 6 and 7 provide comparative data on the specificity of five mAbs raised against swine IgG using an anti-fluorescein mAb as a negative control (Table 2). When the HCAB are directly immobilized, all five mAbs recognized one or more IgG subclasses (Fig. 6). 3H7 and M19 appeared most “polyclonal” but only weakly recognized IgG3. The mAb considered anti-IgG2 specific (34.1.7d; Table 2) recognized nearly all HCABs except for IgG1 but it was of low affinity as judged by the scale of the y-axis. MABs 23.49.1,

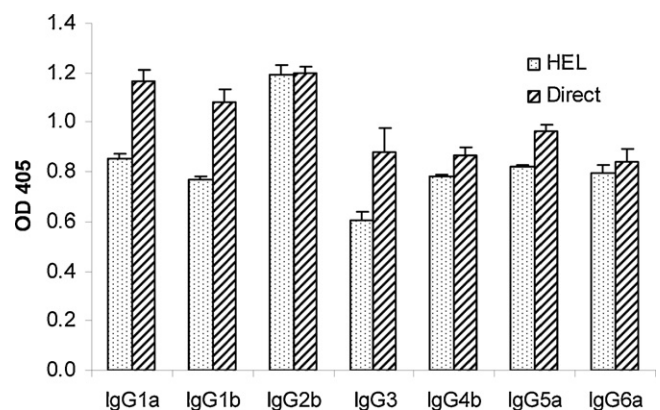


Fig. 5. Antigenic integrity of HCAB when directly adsorbed to microtiter wells at 0.5 µg/well or after binding to HEL-coated plates at 1 µg/ml. Detection was done using biotinylated anti-swine IgG-Fc (B78). The OD 405 values are expressed as mean ± SD after subtraction of a mean background of 0.044.

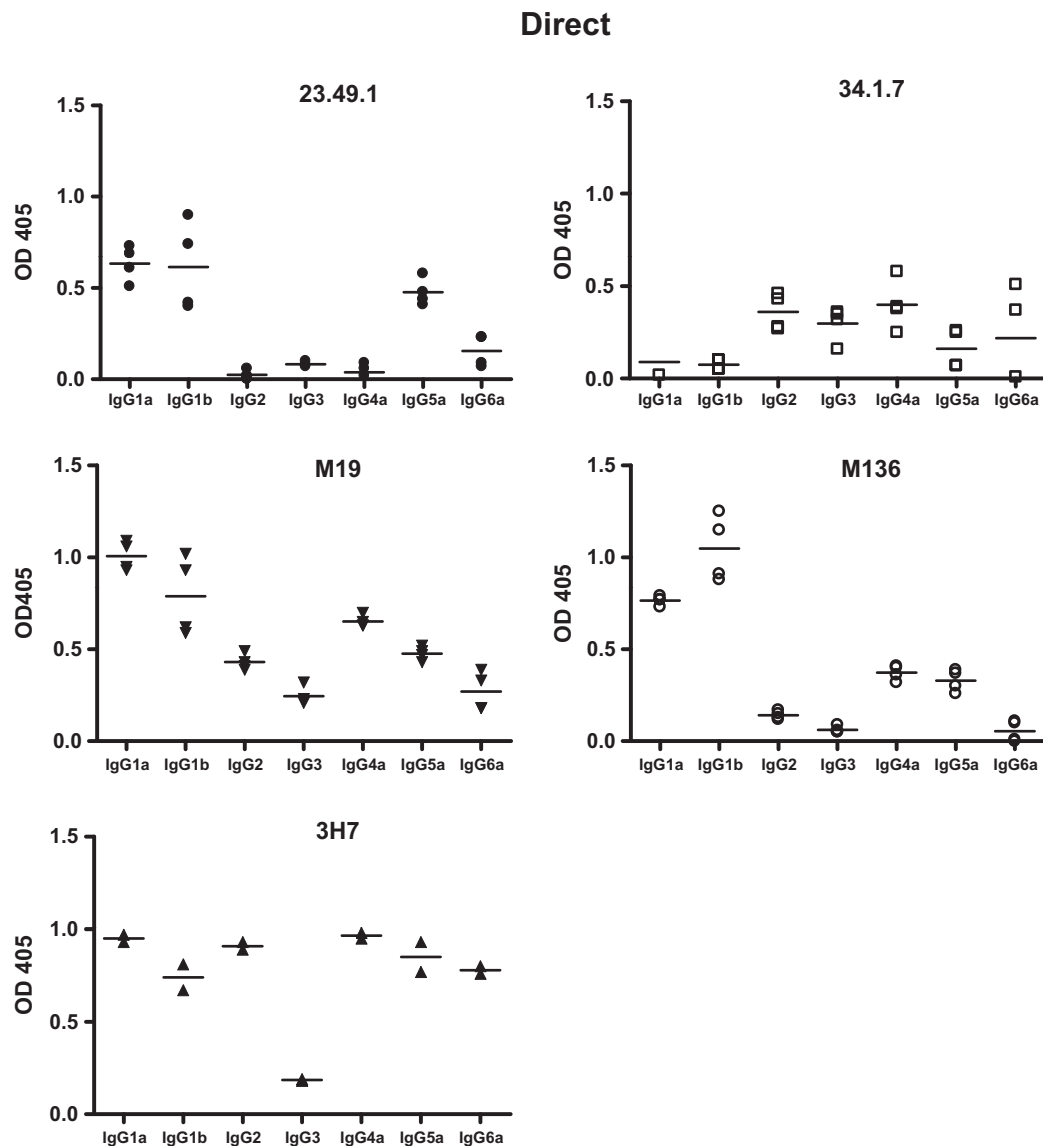


Fig. 6. Scattergram showing the specificity of a panel of five anti-swine IgG mAbs for HCAb directly adsorbed on polystyrene. An anti-fluorescein mAb was used as a control and gave a mean OD 405 of <0.35 which was then subtracted from the mean OD 405 values obtained with the various anti-swine IgG mAbs. Each point represents an independent measurement. The horizontal bar represents the mean. An asterisk indicates those results which differ significantly between IgG1^a and IgG1^b ($p < 0.05$).

M136 and M19 preferentially recognized both allotypes of IgG1. However these mAbs also recognized other IgG subclasses but less well. These three mAbs differed slightly in their bias for IgG1^a versus IgG1^b but the bias was not consistent.

When the specificity of the same mAb panel was tested against HCABs immobilized on their antigen, the specificity pattern changed in most cases (Fig. 7). MAb 23.49.1 remained specific for IgG1 but bias for IgG1^b is apparent. M19 now also shows bias to IgG1^b. 3H7 that was nearly pan-specific for directly adsorbed HCAb (Fig. 6), is strongly biased to IgG1^b and also IgG4^a when these HCABs are bound to lysozyme. M136 that favored IgG1 when HCABs were adsorbed, now appears more pan-specific but with considerable variation. The specificity of 34.1.1d was not changed by the orientation of the HCAb; specificity remains biased against IgG1. When HCAb are bound to HEL they show a greater binding variability.

4. Discussion

Described herein is a method that can provide a solution to an important biotechnical and immunodiagnostic problem. The swine

IgG subclasses are used only as a model. The procedure herein describes the successful *in vitro* production of porcine-camelid chimeric HCAb for swine IgG subclass proteins and their allotypic variants. These *in vitro* products were affinity purified, have the expected molecular structure (Fig. 4) and retained their IgG-specific epitopes (Fig. 5). This proof of concept suggests that the technology described should be successful for the *in vitro* synthesis of subclass and allotypic variants of IgG in any species for which their gene sequences are available. Theoretically, the technology could also be used for IgE and IgD, both of which are present in tiny amount yet their gene sequences are known.

We found that TEL affinity columns containing 7–9 mg of HEL removed all detectable HCAb from 300 ml of culture supernatant, leading to the recovery of 1–2 mg. In ongoing studies this amount (340 µg for two mice) is adequate for the production of mAbs.

As shown (Figs. 5–7) the HCAb produced *in vitro* maintained their Ig-specific epitopes, whether directly adsorbed to plastic or when bound to their antigen. However, the relative specificity of most was dependent on the conformation of the target HCAb. We have previously shown that hydrophobic adsorption on plastic is

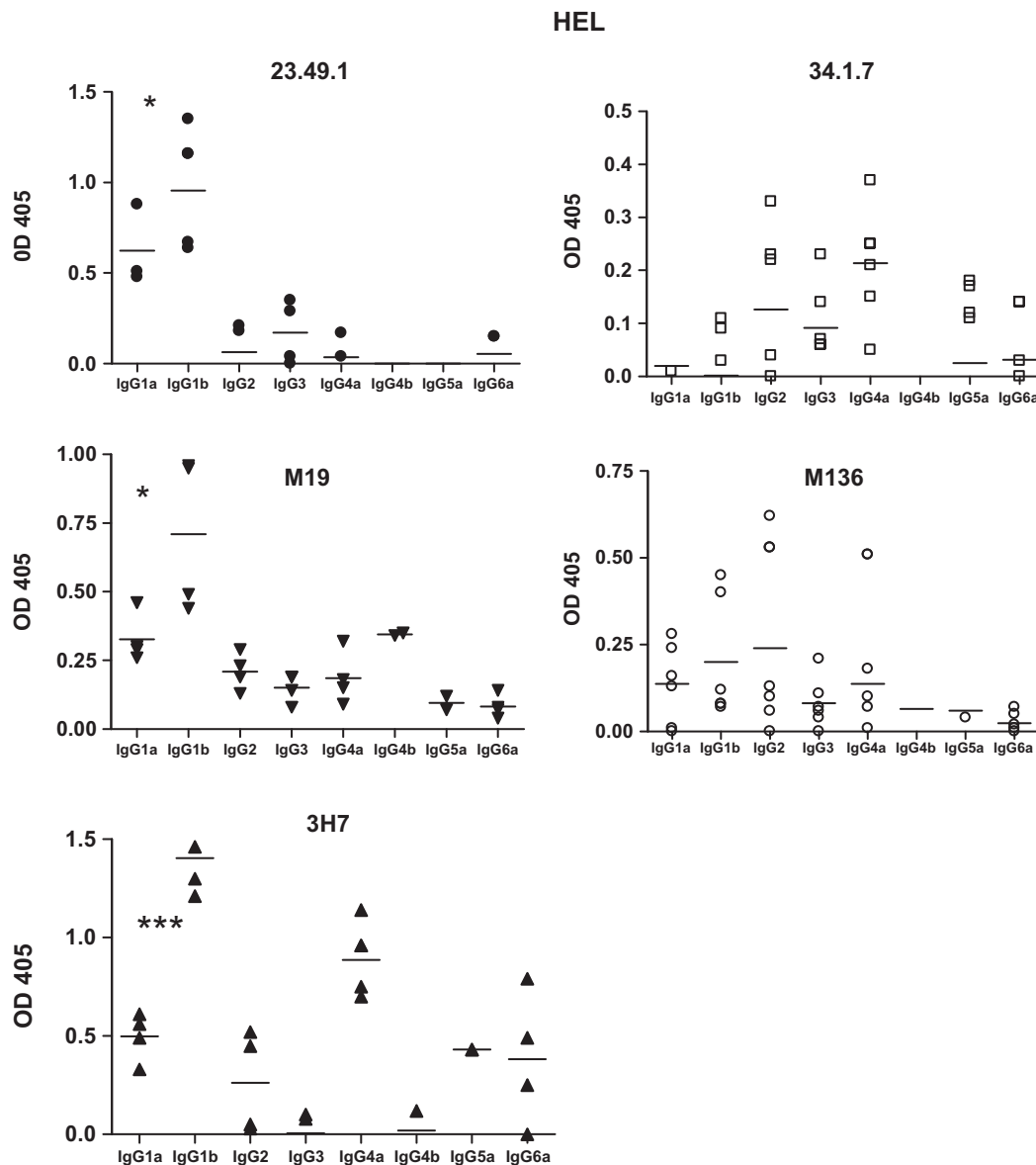


Fig. 7. Scattergram showing the specificity of five anti-swine IgG mAbs for HCAb immobilized by binding to HEL. Other details are the same as in Fig. 4. * ($p < 0.05$), *** ($p < 0.005$).

a protein denaturation process and influences epitope exposure (Butler, 1991; Butler et al., 1993; Butler, 2000; Dierks et al., 1986). While many pAb such as B78 (Fig. 5) recognize epitopes when proteins are immobilized in any configuration, mAb are more sensitive to configuration changes and adsorption-induced denaturation (Butler, 2000). Most often, epitopes are lost through denaturation or steric hindrance (Butler, 1991). In studies reported here the IgG1^b allotope appears to be lost by direct adsorption (Fig. 6 versus Fig. 7). Thus 23.49.1 and 3H7 show little allotype bias when IgG1^a and IgG1^b are directly adsorbed, but are strongly biased to the IgG1^b when bound to antigen. While M19 appears rather pan-specific for adsorbed HCAs, it is specific to IgG1^b when HCAs are immobilized on antigen. Another example is 3H7 that is nearly pan-specific for adsorbed HCAs but strongly specific for IgG1^b and IgG4^a when bound to antigen. On the other hand, 34.1.7d retained its pan-specificity for all but IgG1, although it binds with very low affinity in both cases.

In previous studies on the specificity of polyclonal and mAb to bovine IgG2, we observed that nearly all reagents were biased to an immunodominant epitope in the IgG2^a genetic variant (Butler et al.,

1994; Heyermann and Butler, 1987). Because of these findings, we included a comparison of the specificity of anti-swine IgG reagents for allotypic variants of two swine subclasses; IgG1 and IgG4. We chose these because: (a) IgG1 comprises the majority of the IgG in conventional swine based on transcript frequency (Fig. 2; Butler and Wertz, 2006; Kloep et al., 2012), (b) the two allotypic variants differ significantly in their hinge region sequence and (c) IgG1^b and IgG4^b share the same hinge (Fig. 2). Results presented in Fig. 7 show that when HCAb are immobilized by binding to HEL, three of the mAb tested that recognize IgG1, are biased in favor of IgG1^b. Therefore, we suspected they might recognize the IHQPQT motif in the hinge of IgG1^b. However, IgG4^b shares the same motif so we should have observed similar results. Rather, those mAbs that were IgG1^b-biased (23.49.1; M19; and 3H7) were not biased to IgG4^b and in fact, 3H7, was biased to IgG4^a. Thus, allotypic bias toward IgG1^b is more complex.

In the majority of our tests, IgG3 was often recognized less well than other IgG subclasses. IgG3 is the ancestral IgG for swine and is structurally unique (Fig. 2; Butler et al., 2009). It was overlooked in early studies because it is expressed in fetal/newborn animals

(Butler and Wertz, 2006; Kacskovics et al., 1994; Kloep et al., 2012). The porcine IgG used to prepare B78 and most mAbs used in this study, were purified from adult swine serum which is rich in IgG1 but contains little IgG3 (Fig. 2). Since IgG3 shares the least homology with the other subclasses, the weak activity to the chimeric IgG3 should not be surprising.

Our findings reveal the importance of purified standard for IgG subclasses and their allotypic variant when establishing the specificity of mAb. Since specificity can be conformationally dependent, we believe it is important to determine specificity using IgG bound to its antigen. This should be the same as *in vivo*, and when mAbs are tested in solid-phase immunoassays in which subclass mAbs are tested against the IgG immobilized on its antigen. In most cases, investigators preparing mAb test their hybridoma supernatants against the target protein adsorbed on microtiter wells. Using this procedure, the bias for IgG1^b for HEL-bound HCAb we observed would have been overlooked (Fig. 7). To avoid such a pitfall we engineered our HCAb for uniform specificity to lysozyme so that potential denaturation could be avoided and the subclass HCAb would be in a configuration similar to when bound to antigen *in vivo*.

This study raises a question of what is marketed as a mAb. Theoretically, a single hybridoma clone produces an antibody that recognizes only a single epitope which may of course be shared or partially shared among antigens. Alternatively, the “clone” could be mixed; hybridomas recognizing two or more epitopes. The latter would easily go unrecognized if suitable reference standards for each allotype are not available during the selection process. It is generally accepted that most of the anti-porcine IgG we tested were merely selected because they recognized IgG or differently recognized IgG separated by ion-exchange chromatography as reviewed in the introduction. Our results with the panel of existing anti-IgG mAbs we tested suggest that many may actually be at least oligoclonal. We believe this is because the producer did not have an entire panel of reference standard IgG variants available during the clonal selection process. The strength of the technology is that the panel of reference standards would be available like those in Fig. 2.

It is not our purpose in this report to evaluate the specificity of all available mAbs and pAbs to swine IgG. Rather, we provide a technology for expressing IgG subclass and allotypic variants *in vitro*. Our technology avoids the need for double transfection with light and heavy chain constructs and thus the use of complicated clonal vectors but still delivers subclass proteins. Furthermore, using engineered HCABs with a defined specificity allows the specificity of mAb to be tested against the HCAb immobilized in native configuration.

Acknowledgements

Research supported by USDA-AFRI NIFA/DHS grant 2010-39559-21860. Jessica Jew, University of Iowa, Pat Boyd ANRI-USDA and Ann Kindt, Vrije Universiteit Brussels.

References

- Auchincloss Jr., H., Sachs, D.H., 1998. Xenogeneic transplantation. *Annual Review of Immunology* 16, 433–470.
- Benacerraf, B., Ovary, Z., Block, K.J., Franklin, E.C., 1963. Properties of guinea pig 7S antibodies. I. Electrophoretic separation of two types of guinea pig 7S antibodies. *Journal of Experimental Medicine* 117, 937–949.
- Bleher, D.S., Hicks, A.C., Behr, M., Meteyer, C.U., Berlowski-Zier, B.M., Buckles, E.L., Coleman, J.T.H., Darling, S.R., Gargas, A., Niver, R., Okoniewski, J.C., Rudd, R.J., Stone, W.B., 2009. Bat white-nose syndrome: an emerging fungal pathogen? *Science* 323, 227.
- Bratsch, S., Wertz, N., Chaloner, K., Kunz, T.H., Butler, J.E., 2011. The little brown bat displays a highly diverse VH, DH and JH repertoire but little evidence of somatic hypermutation. *Developmental and Comparative Immunology* 35, 421–430.
- Brown, W.R., Kacskovics, I., Amendt, B.A., Blackmore, N.B., Rothschild, M., Shinde, R., Butler, J.E., 1995. The hinge deletion variant of porcine IgA results from a mutation at the splice acceptor site in the first Cα intron. *Journal of Immunology* 154, 3836–3842.
- Burnett, R.C., Hanly, W.C., Zhai, S.K., Knight, K.L., 1989. The IgA heavy chain gene family in rabbits: cloning and sequence analysis of 13C alpha genes. *EMBO Journal* 8, 4041–4047.
- Butler, J.E., 1969. Bovine immunoglobulins. *Journal of Dairy Science* 52, 1895–1909.
- Butler, J.E., 1983. Bovine immunoglobulins: an augmented review. *Veterinary Immunology* 4, 43–152.
- Butler, J.E., 1991. The behavior of antigens and antibodies immobilized on a solid-phase. In: Van Regenmortel, M.H.V. (Ed.), *Structure of Antigens*, Vol. 1. CRC Press, Boca Raton, FL, pp. 209–258.
- Butler, J.E., 2000. Solid supports in enzyme-linked immunosorbent assay and other solid-phase immunoassays. In: Ngo, T.T. (Ed.), *Developments in Immunoassay Technology, Methods: A Comparison to Methods in Enzymology*. Academic Press, San Diego, CA, pp. 4–23.
- Butler, J.E., 2006. Preface: why I agreed to do this. *DCI* 30, 1–17.
- Butler, J.E., Navarro, P., Heyermann, H., 1994. The heterogeneity of bovine IgG2. VI. The comparative specificity of monoclonal and polyclonal capture antibodies for IgG2a (A1) and IgG2a (A2). *Veterinary Immunology and Immunopathology* 40, 119–133.
- Butler, J.E., Ni, L., Brown, W.R., Joshi, K.S., Chang, J., Rosenberg, B., Voss Jr., E.W., 1993. The immunochemistry of sandwich ELISA. VI. Greater than ninety percent of monoclonal and 75% percent of polyclonal antiluoresceinyl capture antibodies (CABs) are denatured by passive adsorption. *Molecular Immunology* 30, 1165–1175.
- Butler, J.E., Sinkora, M., 2007. The isolator piglet: a model for studying the development of adaptive immunity. *Immunologic Research* 39, 33–51.
- Butler, J.E., Weber, P., Sinkora, M., Baker, D., Schoenherr, A., Mayer, B., Francis, D., 2002. Antibody repertoire development in fetal and neonatal piglets. VIII. Colonization is required for newborn piglets to make serum antibodies to T-dependent and type 2 T-independent antigens. *Journal of Immunology* 169, 6822–6830.
- Butler, J.E., Wertz, N., 2006. Antibody repertoire development in fetal and neonatal piglets. XVII. IgG subclass transcription revisited with emphasis on new IgG3. *Journal of Immunology* 177, 5480–5489.
- Butler, J.E., Wertz, N., Deschacht, N., Kacskovics, I., 2009. Porcine IgG: structure, genetics and evolution. *Immunogenetics* 61, 209–230.
- Butler, J.E., Wertz, N., Zhao, Y., Zhang, S., Bao, Y., Bratsch, S., Kunze, T.H., Whitaker Jr., J.O., Schountz, T., 2011. Two suborders of bats have the canonical isotypes repertoire of other eutherian mammals. *Developmental and Comparative Immunology* 35, 272–284.
- De Genst, E., Saerens, D., Muyldermans, S., Conrath, K., 2006. Antibody repertoire development in camelids. In: Butler, J.E. (Ed.), *Antibody Repertoire Development*. Developmental and Comparative Immunology, Vol. 30, pp. 187–198.
- Dierks, S.E., Butler, J.E., Richerson, H.B., 1986. Altered recognition of surface-adsorbed compared to antigen bound antibodies in the ELISA. *Molecular Immunology* 23, 403–411.
- Dillender, M.A., 1988. The immune response of swine to phosphorylcholine. Ph.D. Thesis, University of Iowa.
- Engvall, E., Perlmann, P., 1972. Enzyme-linked immunosorbent assay, ELISA. *Journal of Immunology* 109, 129–135.
- Greenberg, A.S., Avila, D., Hughes, M., Hughes, A., McKinney, E.C., Flajnik, M.F., 1995. A new antigen receptor gene family that undergoes rearrangement and extensive diversification in sharks. *Nature* 374, 168–173.
- Hamers-Castermann, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman, N., Hamers, R., 1993. Naturally occurring antibodies devoid of light chains. *Nature* 363, 446–448.
- Heyermann, H., Butler, J.E., 1987. The heterogeneity of bovine IgG2. IV. Structural differences between IgG2a bearing the A1 and A2 allotypic determinants. *Molecular Immunology* 24, 1327–1334.
- Kacskovics, I., Sun, J., Butler, J.E., 1994. Five subclasses of swine IgG identified from the cDNA sequences of a single animal. *Journal of Immunology* 153, 3566–3573.
- Kaltreider, H.B., Johnson, J.S., 1972. Porcine immunoglobulins I. Identification and subclasses and preparation of specific antisera. *Journal of Immunology* 109, 992–998.
- Kehoe, J.M., Capra, J.D., 1974. Nature and significance of immunoglobulin subclasses. *New York State Journal of Medicine* 74, 489–491.
- Kilian, M., Russell, M.W., 2004. Microbial evasion of IgA function. In: Mestecky, J., Lamm, M.E., Strober, W., Bienenstock, J., McGhee, J.R., Mayer, L. (Eds.), *Mucosal Immunology*. Elsevier/Academic Press, Amsterdam, pp. 291–303.
- Kloep, A., Wertz, N., Mendicino, M., Ramsoondar, J., Butler, J.E., 2012. Linkage haplotype for IgG and IgA subclass genes. *Immunogenetics*, PMID: 22350166.
- Kunkel, H.G., 1963. Myeloma proteins and antibodies. In: *Harvey Lecture Series* 59. Academic Press, N.Y., pp.219–242.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lefranc, M-P., Lefranc, G., 2001. *The Immunoglobulin Facts Book*. Academic Press, NY, p. 457.
- Lemke, C.D., Haynes, J.S., Spaete, R., Adolphson, D., Vorwald, A., Lager, K., Butler, J.E., 2004. Lymphoid hyperplasia resulting in immune dysregulation is caused by PRRSV infection in pigs. *Journal of Immunology* 172, 1916–1925.
- Lorusso, A., Vincent, A.L., Gramer, M.R., Lager, K.M., Ciacci-Zanella, J.R., 2012. Contemporary epidemiology of North American lineage triple reassortant influenza A viruses in pigs. *Current Topics in Microbiology and Immunology*, PMID: 22266673.

- Mendicino, M., Ramsoondar, J., Phelps, C., Vaught, T., Ball, S., LeRoith, T., Monahan, J., Chen, S., Dandro, A., Boone, J., Jobst, P., Vance, A., Wertz, N., Bergman, Z., Sun, X.-Z., Polejaeva, I., Butler, J., Dai, Y., Ayares, D., Wells, K., 2011. Generation of antibody- and B cell-deficient pigs by targeted disruption of the J-region gene segment of the heavy chain locus. *Transgenic Research* 20, 625–641.
- Metzger, J.J., Fougereau, M., 1967. Characterization of two subclasses of gamma G immunoglobulin in swine. *Comptes rendus des séances de l'Académie des sciences. Série P, Sciences naturelles* 265, 724–727.
- Mossmann, T.R., Coffman, R.L., 1989. TH1 and TH2 cells: different patterns of lymphokine secretion leads to different functional properties. *Annual Review of Immunology* 7, 145–173.
- Mullens, M.A., Register, K.B., Bayles, D.O., Butler, J.E., 2011. *Haemophilus parasuis* exhibits IgA protease activity but lacks homologs of the IgA protease genes of *Haemophilus influenzae*. *Veterinary Microbiology* 153, 407–412.
- Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J.A., Hamers, R., 1994. Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. *Protein Engineering* 7, 1129–1135.
- Nguyen, V.K., Hamers, R., Wyns, L., Muyldermans, S., 2000. Camel heavy-chain antibodies: diverse germline V_HH and specific mechanisms enlarge the antigen-binding repertoire. *EMBO Journal* 19, 921–930.
- Plaut, A.G., Gilbert, J.V., Artenstern, M.A., Capra, J.D., 1975. *Neisseria gonorrhoeae* and *Neisseria meningitidis*: extracellular enzyme cleaves human immunoglobulin A. *Science* 193, 1103–1105.
- Potter, M., Boyce, C., 1962. Inductions of plasma-cell neoplasms in strain BALB/c mice with mineral oil and mineral oil adjuvants. *Nature* 193, 1086–1087.
- Prokesova, L., Rejnek, J., 1973. Molecular heterogeneity of newborn piglet IgG. *Immunochimistry* 10, 607–609.
- Ramsoondar, J., Mendicino, M., Phelps, C., Vaught, T., Ball, S., Monahan, J., Chen, S., Dandro, A., Boone, J., Jobst, P., Vance, A., Wertz, N., Polejaeva, I., Butler, J., Dai, Y., Ayares, D., Wells, K., 2011. Targeted disruption of the porcine immunoglobulin kappa light chain locus. *Transgenic Research* 20, 643–653.
- Rapacz, J., Hasler-Rapacz, J., 1982. Immunogenetic studies on polymorphism, post-natal acquisition and development of immunoglobulin gamma. [IgG] in swine. In: *Proceeding of the 2nd World Congress on Genetics Applied to Livestock Production*. VIII, Editorial Garsi, Madrid.
- Ravetch, J.V., Kinet, J.-P., 1991. Fc receptors. *Annual Review of Immunology* 9, 457–492.
- Rogers, C.S., Stoltz, D.A., Meyerholz, D.K., Ostedgaard, L.S., Rokhlina, T., Taft, P.J., Rogan, M.P., Pezzulo, A.A., Karp, P.H., Itani, O.A., Kabel, A.C., Wohlford-Lenane, C.L., Davis, G.J., Hanfland, R.A., Smith, T.L., Samuel, M., Wax, D., Murphy, C.N., Rieke, A., Whitworth, K., Uc, A., Starner, T.D., Brogden, K.A., Shilyansky, J., McCray Jr., P.B., Zabner, J., Prather, R.S., Welsh, M.J., 2008. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science* 321, 1837–1841.
- Spieker-Polet, H., Yam, P.-C., Knight, K.L., 1993. Differential expression of 13 IgA-heavy chain genes in rabbit lymphoid tissues. *Journal of Immunology* 150, 5457–5465.