ACQUIRED SELECTIVE IgA DEFICIENCY INDUCED BY DIETARY BOVINE IgA

by

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ABSTRACT OF THE THESIS

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Selective IgA deficiency (sIgAD) is the most common immunodeficiency in humans. Auto-reactive antibodies to human IgA are found in the serum of 20-40% of individuals with sIgAD. It is unknown whether these antibodies play a role in the pathogenesis of this immunodeficiency, and most researchers believe that they are secondary to the onset of sIgAD. However, it is possible that in these individuals, the anti-IgA antibodies are in fact responsible for the removal of IgA from serum, and are originally generated against xenogeneic IgA. To examine this hypothesis, the presence of anti-bovine and anti-human IgA antibodies was tested by ELISA in serum samples from IgA-deficient and control individuals. All 14 of the IgA-deficient individuals that were tested had IgG anti-bovine IgA antibodies (100%), whereas only 8 had IgG anti-human IgA antibodies (57%). Individuals with both anti-bovine and anti-human IgA antibodies always had a higher titre against bovine IgA than against human IgA. Of 18 control individuals who have normal serum levels of IgA and no anti-human IgA antibodies, a surprisingly high proportion (61%) had IgG anti-bovine IgA antibodies in their serum. These results strongly support the hypothesis

that the anti-human IgA antibodies found in IgA-deficient individuals are originally produced against xenogeneic IgA, specifically bovine IgA found in dietary beef products. These antibodies can be found in many normal individuals, but only those that cross-react with endogenous human IgA will lead to the removal of IgA from circulation, and to sIgAD. Thus, sIgAD with anti-IgA antibodies is an acquired immunodeficiency, initiated by cross-reactive antibodies consumed in the diet.

ACKNOWLEDGMENTS

DEDICATION

In loving memory of Savta Hana.

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INTRODUCTION

Selective IgA deficiency

Immunoglobulin A (IgA) serves as the first line of defense against foreign antigens encountered along mucosal surfaces. Secretory IgA in the mucosa is mostly dimeric and is covalently bound to a secretory component which facilitates its secretion and protects it from proteolytic digestion. Serum IgA is found mainly in the monomeric form (1). Selective IgA deficiency (sIgAD) is the most common immunodeficiency in man. It is usually defined as a serum IgA concentration lower than 0.05 mg/ml (normal range: 0.8 - 4.5 mg/ml), with normal serum levels of IgG, IgM, IgD and IgE. Affected individuals are also deficient in secretory IgA antibodies (reviewed in (2-4)). In Western populations the frequency of sIgAD among healthy blood donors is reported to be between 1:328 and 1:1,428 (5-11). The large variation in frequency is mostly due to the different techniques used to determine serum IgA levels, including radial immunodiffusion, passive haemagglutination, radioimmunoassay and enzyme-linked immunosorbent assays (ELISA) which differ dramatically in sensitivity.

The clinical manifestations of sIgAD are highly variable. Most affected individuals are clinically asymptomatic and the condition is often first diagnosed during screening of donated blood with no follow-up. Those individuals who do develop symptoms tend to suffer from recurrent upper and lower respiratory tract infections, gastro-intestinal diseases including acute and chronic diarrheal illness, and various other infections of the skin and mucosal surfaces (3). A long term follow-up study of healthy, randomly selected, adult blood donors with sIgAD was documented by Koskinen *et al.* over a period of 20 years in Finland (12). Compared to people with low or normal levels of serum IgA, an increased number of

IgA-deficient adults suffered from severe infections such as bronchitis and pneumonia, recurrent respiratory and urinary tract infections, drug allergies, atopic diseases such as asthma, conjunctivitis and eczema, and milk intolerance. Additionally, this IgA-deficient population was more prone to developing autoimmune diseases, the most common of which was seropositive rheumatoid arthritis.

The molecular basis of sIgAD is not known, but is likely to differ among affected individuals. Recent studies have linked the pathogenesis of sIgAD to defects in the IgA production mechanism, including defects in isotype switching from IgM-producing B cells into plasma cells that secrete IgA, problems in terminal differentiation of the plasma cells, genetic abnormalities specific to the IgA synthesis pathway, or gene regulation defects (13-16). In one reported case, sIgAD was transferred to an HLA-matched sibling in a bone-marrow transplant, with both donor and recipient having normal serum levels of IgG (17), suggesting a defect of haematopoietic origin. However, in the majority of IgA-deficient individuals, the reason for the IgA deficiency remains elusive.

The variable incidence of sIgAD in different populations, as high as 1:328 affected in some Western populations (10) compared to as low as 1:18,500 affected Japanese (18), indicates that genetic background is a contributing factor at least in some cases of sIgAD. This is also evident from several family studies in which numerous members of the same family developed sIgAD (19-21). Several gene associations have been suggested, such as HLA loci of both class I and class II, either alone or in combination. Some of these are HLA-A1, A2, A28, B4, B8, B14, B40, DR1, DR3, DR7, A1/B14, A28/B14 and A1/B8 (Summarized in (22)). In 2005, a missense mutation in the TACI gene, which encodes a tumor necrosis factor receptor family member, was reportedly associated with symptomatic

sIgAD and common variable immunodeficiency (23). However, the mutation was only found in 1 of 16 IgA-deficient patients tested in that particular study (compared with none of the 50 normal controls). Therefore, further studies are needed in order to confirm this association. Moreover, it was reported that sIgAD may occur in only one identical twin, suggesting that environmental factors can also play a significant role in the pathogenesis of this condition (19).

Anti-IgA antibodies in IgA-deficient individuals

Since the 1960s, a host of autoimmune phenomena have been reported in association with sIgAD, including antibodies to self IgA (24). Anti-IgA antibodies were first observed by Fudenberg *et al.* in patients with ataxia telangiectasia and dysgammaglobulinemia who lacked serum IgA, using the haemagglutination inhibition method (25). In the early 1970s it became apparent that a significant number of IgA-deficient individuals have high titres of auto-antibodies specific for human IgA. The prevalence of anti-IgA antibodies amongst people with sIgAD is reported to be between 20-40% depending on the study and method of detection employed. A survey of the most significant reports in the literature is presented in *Table 1*.

The variation in percentages observed across studies may be attributed to the genetic differences in the cohorts of patients chosen for a particular study, and/or to the sensitivity of the method used to detect the presence and titre of anti-IgA antibodies. In 1983, Hammarstrom *et al.* observed that the presence of anti-IgA antibodies in sIgAD seems to be correlated with HLA-DR3 (30). This finding was further analyzed by Strothman *et al.* who identified only a weak association with DR3. Instead, Strothman's group found an increased

frequency of DR7 among IgA-deficient individuals with anti-IgA antibodies, alongside an increased frequency of DR1 among those without the antibodies (22).

TABLE 1: Summary of reports of anti-IgA antibodies in IgA-deficient individuals

Authors (Reference)	Method used to detect anti-IgA	No. of samples tested	No. of individuals with anti-IgA antibodies (*)
Sandler et al. 1994 (26)	PHA[†]	80[a] 87[b] 358[c]	61 (76%) 27 (31%) 97 (27%)
Laschinger et al. 1984 (27)	PHA	21	12 (57%)
Thibault et al. 2006 (28)	ELISA	73	39 (53%)
Nadorp et al. 1973 (29)	Binding capacity of serum to IgA-cellulose	16	7 (44%)
Hammarstrom et al. 1983 (30)	ELISA	33	14 (42%)
Ammann & Hong 1971 (31)	PHA	30	12 (40%)
Ferreira et al. 1988 (32)	ELISA	46	17 (37%)
Clark et al. 1983 (10)	PHA	19	7 (37%)
Bjorkander et al. 1987 (33)	PHA & ELISA	185	53 (29%)
Munks et al. 1998 (34)	PHA	479	142 (29%)
Wells et al. 1980 (8)	PHA	11	3 (27%)
Koistinen et al. 1977 (35)	PHA	156	39 (25%)
Koskinen et al. 1995 (36)	ELISA	159	30 (19%)
Holt et al. 1977 (7)	PHA	34	6 (18%)
Petty et al. 1979 (37)	PHA	27	5 (18%)
Vyas et al. 1975 (6)	PHA	83	13 (15%)
Koistinen & Sarna 1975 (38)	PHA & RIA[‡]	163	15 (9%)
Pai et al. 1974 (39)	PHA	12	1 (8.3%)

* percentage of anti-IgA antibody positive individuals; [†] PHA - passive haemagglutination; [‡] RIA - radioimmunoassay; [a] IgA-deficient patients who had transfusion reaction; [b] IgA-deficient blood donors; [c] healthy IgA-deficient individuals.

The role of anti-IgA antibodies in the etiology of sIgAD and the antigenic trigger for their production are both controversial. It remains unclear whether the presence of anti-IgA antibodies in individuals with sIgAD are a consequence of the IgA-deficient state or the cause of the deficiency. In earlier studies, various investigators suggested that anti-IgA antibodies are secondary to the state of IgA deficiency and offered several theories why that could be the case. For example, it could be argued that the congenital lack of IgA makes this molecule a non-self molecule and therefore these individuals will be more prone to make anti-IgA antibodies if exposed to human IgA or to a cross-reactive xenogeneic IgA. It was also suggested that the lack of IgA may facilitate the entry of food antigens including foreign IgA through the intestinal mucosa (29;40). Exposure to human IgA in blood transfusions (41), immunization of the mother to foetal IgA during delivery (42), foetal exposure to maternal IgA via damaged placental villi *in utero* (42), sensitization to colostral IgA while breast feeding (37), and reaction to small but undetectable amounts of serum or cell surface IgA (37) were also suggested as possible sources for the priming antigen. However, none of these theories have any supportive evidence and in fact are contrary to the available statistical data on sIgAD. This issue is discussed in detail in the Discussion section.

The presence of anti-IgA antibodies is not always an indication for sIgAD. Anti-IgA antibodies of the IgM isotype ('natural antibodies') or anti-IgA antibodies with limited specificity (which include subclass-specific, allotype-specific, Fab-specific antibodies, etc.) may occur in normal individuals with normal or low levels of serum IgA (43). The anti-IgA antibodies in individuals with a <u>complete</u> absence of IgA are exclusively class-specific, mostly of the IgG1 isotype, and by definition react with both IgA subclasses 1 and 2 (30;43).

We propose the hypothesis that the class-specific anti-IgA antibodies that occur in IgA-deficient individuals are in fact the cause of the deficiency, and that these antibodies are actually cross-reactive antibodies to xenogeneic IgA, specifically bovine IgA, originating in food. This hypothesis was inspired by an IgA-deficient patient with serum anti-IgA antibodies who presented some interesting blood test results. This patient found that upon removal of non-Kosher beef from the diet, there was an appreciable improvement in health. Remarkably, there was also a concordant reduction observed in the serum auto-antibody level detected in blood tests. Further reduction occurred after beginning a diet which consisted only of poultry and fish, entirely eliminating all red meats and dairy products. Since Kosher beef contains considerably less blood and therefore less bovine IgA than does non-Kosher beef, it seemed likely that consumption of xenogeneic IgA from beef products may be the trigger for anti-IgA auto-antibody production. One of the stipulations from this hypothesis is that most or all individuals with sIgAD that have high enough (> 1:250) titres of anti-human IgA antibodies will also have a high titre against bovine IgA, which can be readily tested. Moreover, this hypothesis also implies that many normal individuals will have anti-bovine IgA antibodies but no anti-human IgA antibodies. This hypothesis led to the current investigation of the presence of cross-reactive antibodies in the sera of IgA-deficient individuals.

MATERIALS & METHODS

Serum samples

IgA-deficient (IgAD) sera #1-13 were generously provided by Michelle Henry of the American Red Cross, in Philadelphia, PA. Absence of IgA was determined at the Red Cross facility, by ELISA. Each serum sample was also tested for the presence or absence of anti-human IgA antibodies by haemagglutination, at the same facility. Patient #14 is the IgA-deficient patient who approached the laboratory. The IgAD #14 serum sample used in this study was drawn on 12/05 from patient #14, and kindly provided by Dr. Henry Homburger (University of Michigan, Ann Arbor, MI). This sample was tested at the Mayo Clinic, Rochester, MN, and found to have an IgG anti-IgA level of 436 (expressed as % of negative control), by Immunoradiometric Assay (44). Control sera were obtained from volunteers through the RWJMS-UMDNJ facility in New Brunswick, NJ. All of the control sera had normal immunoglobulin profiles. All sera were aliquoted and kept at -90°C until use. Sera were kept at 4°C after thawing.

Purification of bovine IgA

Bovine IgA was purified on an affinity column from pooled ox serum purchased from Colorado Serum (Denver, CO). The affinity column was made by conjugating sheep anti-bovine IgA polyclonal antibodies (Bethyl, Montgomery, TX) to CNBr-activated Sepharose 4B (Amersham Biosciences, Piscataway, NJ). Bovine IgA was eluted using glycine-HCl buffer (0.2 M glycine, pH 2.8). The purity of each eluted fraction was assessed by SDS-PAGE. Eluates were combined, dialyzed against phosphate-buffered saline (pH 7.4)

and concentrated using a 63.5 mm Ultracel ultrafiltration membrane (Millipore, Billerica, MA). Protein concentration was determined by spectrophotometry: absorbance of 1.4 at 280 nm equals 1.0 mg of IgG. Aliquots were stored at -90°C.

Purification of mouse monoclonal antibodies

Mouse monoclonal anti-human IgG Fc was previously purified and analyzed in our laboratory from hybridoma clone HP6017 (ATCC, Manassas, VA). Aliquots were lyophilized and stored at -90°C.

Mouse monoclonal anti-human IgA1+2 from hybridoma clone CH-EB6.8 (ATCC number HB-200) was a kind gift of Dr. Hiromi Kubagawa (Division of Developmental and Clinical Immunology University of Alabama at Birmingham). This clone's isotype is IgG1 and it is specific for human IgA of both subclasses 1 and 2. Antibodies were purified on Protein A column. Binding of the IgG1 isotype antibodies was done in high salt buffer (3.3 M NaCl, 0.1 M Sodium Borate, pH 8.9) according to (45). Antibodies were eluted in glycine-HCl buffer. The purity of eluted antibodies was assessed by SDS-PAGE. Eluate was dialysed against PBS and the concentration was determined by extinction coefficient: absorbance of 1.4 at 280 nm equals 1.0 mg of IgG. Aliquots were lyophilized and stored at -90°C.

Electrophoresis

For SDS-PAGE analysis, proteins were heated, reduced and run on Tris-HCl gel (Bio-Rad, Hercules, CA). Dual colour Precision Plus molecular weight marker was purchased from Bio-Rad. Human milk lacking in Igs and containing free secretory component (~80kD) was obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). Human IgA,

and bovine and human IgM were purchased from Sigma-Aldrich (St. Louis, MO). Bovine and human IgG were purchased from Equitech-Bio (Kerrville, TX). BSA (~66kD) was purchased from Sigma-Aldrich.

Enzyme-linked immunosorbent assay (ELISA)

Desiccated Immulon 4 HBX strips (Thermo Scientific, Milford, MA) were coated with the relevant antigen diluted in coating buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, 0.02% NaN_3, pH 10.0). They were left at room temperature (RT) for 1-2 hours, followed by overnight (ON) incubation at 4°C. The plates were then blocked using 0.2 ml 2% fish gelatin (Sigma-Aldrich) in PBS. Plates were washed 4 times with PBST (PBS with 0.05% Tween 20). Primary antibodies or serum sample was then added and plates were incubated for 1 hour at RT, followed by a wash step. Alkaline phosphatase (AP)-conjugated secondary antibodies were diluted 1:5,000 in PBST, 0.1 ml was added to the wells and incubated for 1 hour at RT, followed by another wash step. Lastly, 0.1 ml substrate (1 *p*-Nitrophenyl phosphate disodium hexahydrate tablet dissolved in 25 ml 1 M Diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) was added to each well. The colour intensity was quantified by reading the absorbance with an ELISA reader at 405nm after addition of substrate.

Detection of IgG and IgA in human sera by ELISA

Plates were coated in duplicate with 0.1 ml sera diluted 1:500 in coating buffer. After blocking, mouse monoclonal antibodies against human IgG or human IgA were serial three-fold diluted in PBST and 0.1 ml was added starting at a dilution of 1:3,000. AP-conjugated goat anti-mouse Fcγ secondary antibodies (Jackson ImmunoResearch, West

Grove, PA) were diluted 1:5,000 in PBST, and 0.1 ml was added to the wells.

Detection of IgG anti-human IgA antibodies and IgG anti-bovine IgA antibodies in human sera by ELISA

Plates were coated in triplicate with 3 µg/ml human IgA (Biodesign, Saco, ME) or bovine IgA in coating buffer. After blocking, human sera were serial two-fold diluted in PBST and 0.1 ml was added starting at a dilution of 1:250. AP-conjugated goat anti-human Fcγ secondary antibodies (Jackson ImmunoResearch) were diluted 1:5,000 in PBST, and 0.1 ml was added to the wells.

RESULTS

Titres of IgG anti-IgA antibodies are reduced following elimination of red meat products from the diet

As mentioned in the Introduction, this study was inspired by a clinically symptomatic IgA-deficient patient with high titres of anti-IgA antibodies (IgAD sample #14) who decided to test whether switching to consumption of Kosher or no beef, thereby reducing exposure to IgA, would lead to improvement of clinical symptoms. As the patient was being treated continuously at the same clinic over a long period of time, it was possible to obtain blood test results at various time points and to correlate any notable differences in anti-IgA levels to changes in the diet. *Figure 1* shows the patient's level of IgG anti-IgA antibodies
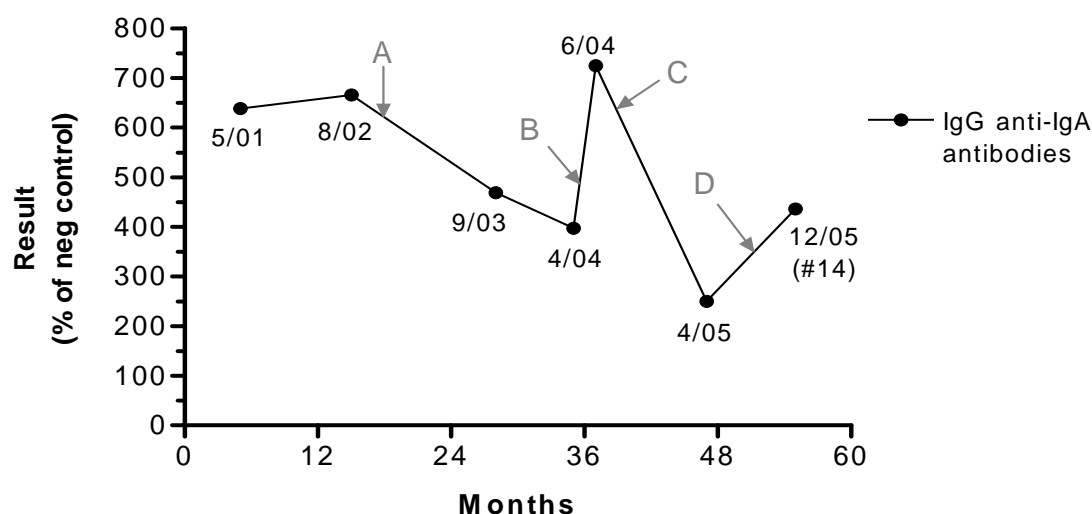
Figure 1. Changes of IgG anti-IgA levels in patient #14 over time. The blood samples were all tested at the Mayo Clinic, Rochester MN using an immunoradiometric assay (IRMA). Results are expressed as % of negative control: 0 - 150 negative; 151-200 limited specificity or low-titre class-specific antibodies; > 200 class-specific antibodies or high-titre limited-specificity antibodies. Changes in the patient's diet are shown at approximate time points: A – switched to kosher red meat (incl. beef, veal, lamb); B – resumed non-kosher red meat intake; C - began poultry diet, with no red meat or dairy products; D – resumed dairy intake, with no red meat.

alongside the changes in dietary intake. The values were obtained using a commercial immunoradiometric assay (44). This assay is similar to a capture ELISA assay, in that IgA-coated beads are reacted with serum, washed and then mixed with radioactively labeled anti-IgG antibodies. The amount of IgG anti-IgA antibodies in the serum correlates to the amount of radioactivity bound to the beads. The results are expressed as per cent difference in arbitrary radioactive units as compared to the number obtained from a negative control (normal serum). The patient's initial levels were 638 (5/2001) and 666 (8/2002) per cent of negative control. After the blood test of 8/2002, the patient removed non-Kosher red meat (including beef, veal and lamb) from the diet (*Figure 1*, time point A). This brought about an improvement in health, as well as a concordant reduction in anti-IgA levels to 469% (9/2003) and 397% (4/2004). After the patient resumed consumption of non-Kosher meat (*Figure 1*, time point B), in the next blood test only two months later, the anti-IgA level increased to 725% (6/2004), considerably higher than the initial levels recorded. This prompted the patient to begin a diet which consisted solely of poultry and fish, entirely eliminating all red meats and dairy products (*Figure 1*, time point C). This change was followed by the most dramatic reduction in anti-IgA levels, to 250% (4/2005). During the following eight months, the patient consumed poultry, still without any red meat. However, dairy products were re-introduced prior to the next blood test (*Figure 1*, time point D). The final blood test on 12/2005 (labeled as IgAD sample #14 in this study) resulted in an elevated anti-IgA level of 436%. The significant reduction observed in the patient's anti-IgA levels following the removal of red meat products from the diet, prompted us to investigate whether bovine IgA may be responsible for inducing the formation of anti-human IgA antibodies found in IgA-deficient serum.

Purification of bovine IgA from ox serum

In order to assess the response to bovine IgA in IgA-deficient patients and normal controls, it was necessary to obtain pure bovine IgA. Since it was commercially unavailable, we purified bovine IgA in large quantities from ox serum as described above in Materials & Methods. Pooled ox serum was run on an affinity column which contained polyclonal purified sheep anti-bovine IgA antibodies. The bovine IgA that was obtained from the column was assessed further by resolving it on SDS-PAGE alongside bovine IgG and IgM, and human IgA, IgG, and IgM (*Figure 2*).
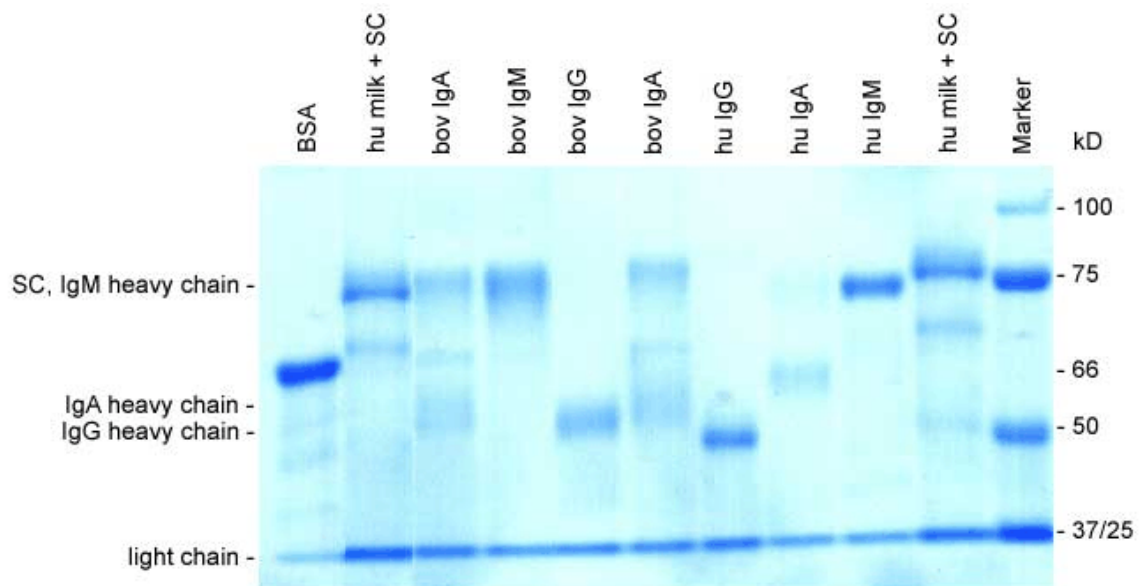
Figure 2. SDS-PAGE comparing bovine Igs and human Igs. Proteins were reduced and run on 7.5% Tris-HCl gel. Positions of free secretory component (SC), heavy chains and light chains are indicated on the left. Molecular weights (kD) are shown alongside the marker on the right.

Determination of IgA and IgG levels in IgA-deficient and control sera

In order to validate that the IgA-deficient sera obtained from the American Red Cross completely lacked IgA, the level of IgA in all sera used in this study was determined by direct ELISA using monoclonal anti-human IgA antibodies. Mouse monoclonal antibodies specific for human IgA of both subclasses 1 and 2 were purified from hybridoma clone CH-EB6.8 (ATCC number HB-200) on a protein A column, as described in Materials & Methods. Purity was analyzed by SDS-PAGE (*Figure 3*). Monoclonal antibody HP6017 (specific to human IgG) was used to confirm that the serum samples were not deficient in IgG. It was purified and analyzed previously in our laboratory. Serum samples from 14 IgA-deficient individuals and 18 controls were tested for levels of IgA and IgG by ELISA, as described in Materials & Methods. Serum samples were bound to the plates at a dilution of 1:500. IgA and IgG were detected using mouse monoclonal antibodies to human IgA and human IgG, respectively. Goat anti-mouse secondary antibodies conjugated to AP were used to detect the monoclonal antibodies. Absorbance was recorded at 405nm after addition of substrate.

Figure 3. SDS-PAGE of purified monoclonal mouse anti-human IgA1+2. Sample was reduced and run on 15% Tris-HCl gel alongside the marker. Molecular weights (kD) are shown on the left.

Table 2 depicts the results of experiments in which the complete absence of IgA in the IgA-deficient sera is verified.

TABLE 2: IgA and IgG levels of IgA-deficient and control sera

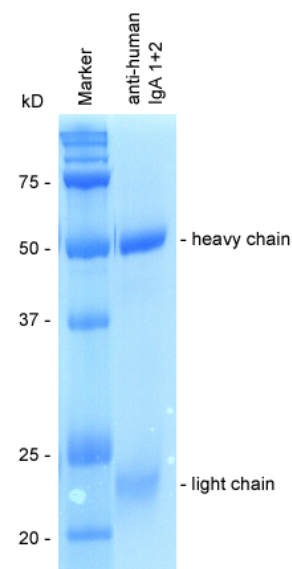| Serum no.	IgA*	IgG*	Serum no.	IgA*	IgG*
Control #1	0.434	0.895	IgAD #1	0.009	1.446
Control #2	0.931	1.383	IgAD #2	0.000	1.307
Control #3	0.385	0.772	IgAD #3	0.005	1.519
Control #4	0.620	1.054	IgAD #4	0.000	0.179
Control #5	0.925	1.909	IgAD #5	0.003	1.411
Control #6	1.576	2.493	IgAD #6	0.002	1.559
Control #7	0.893	1.588	IgAD #7	0.000	1.617
Control #8	1.305	2.320	IgAD #8	0.002	1.247
Control #9	1.448	1.309	IgAD #9	0.004	1.427
Control #10	0.914	2.080	IgAD #10	0.000	1.453
Control #11	1.024	2.130	IgAD #11	0.002	1.349
Control #12	1.403	2.323	IgAD #12	0.005	1.686
Control #13	0.626	1.182	IgAD #13	0.000	1.680
Control #14	0.503	1.099	IgAD #14	0.002	1.639
Control #15	1.009	1.172			
Control #16	0.271	1.457			
Control #17	0.800	1.043			
Control #18	0.546	1.304			
Pooled serum †	0.647	1.475			

* OD_{405nm} recorded using 1:3000 dilution of monoclonal antibodies against human IgA or IgG (mean of duplicate wells, SD was always < 20%). Results are shown 30 mins after addition of substrate. Mean background values of secondary antibody interaction with each serum sample were deducted from the mean experimental values. The raw mean background values ranged from 0 - 0.046.
† Pooled normal human serum was purchased from Sigma-Aldrich.

As expected, none of the IgA-deficient samples have any IgA, whereas IgA can be found in

all of the normal control sera. IgG levels were normal in both IgA-deficient and control sera.

One serum sample (IgAD #4) had very low levels of both IgA and IgG which could indicate

a common variable immunodeficiency or a similar condition rather than sIgAD. Interestingly, this individual had an appreciable titre of IgG anti-bovine IgA antibodies (*Figure 4* and *Table 3*).

Anti-bovine but not anti-human IgA antibodies are present in all IgA-deficient individuals

Fourteen IgA-deficient serum samples were assayed by a direct ELISA for the presence of IgG anti-human IgA antibodies and IgG anti-bovine IgA antibodies. As described in Materials & Methods, antigen (human or bovine IgA) was bound to 96-well plates and probed with serial dilutions of human serum in triplicate. Levels of human IgG were detected by AP-goat anti-human Fcγ secondary antibodies. Absorbance was recorded at 405nm after addition of substrate.

Figure 4 shows the levels of IgG anti-human IgA (clear bars) and IgG anti-bovine IgA (dark bars) in IgA-deficient individuals. Results are shown as absorbance obtained at each dilution of serum (mean of triplicates). Antibody titres for each IgA-deficient serum are presented in *Table 3*. The antibody titre is defined here as the last serum dilution that recorded an absorbance (mean of triplicates) higher than 0.2. This cutoff is marked on each graph by a vertical dashed line.
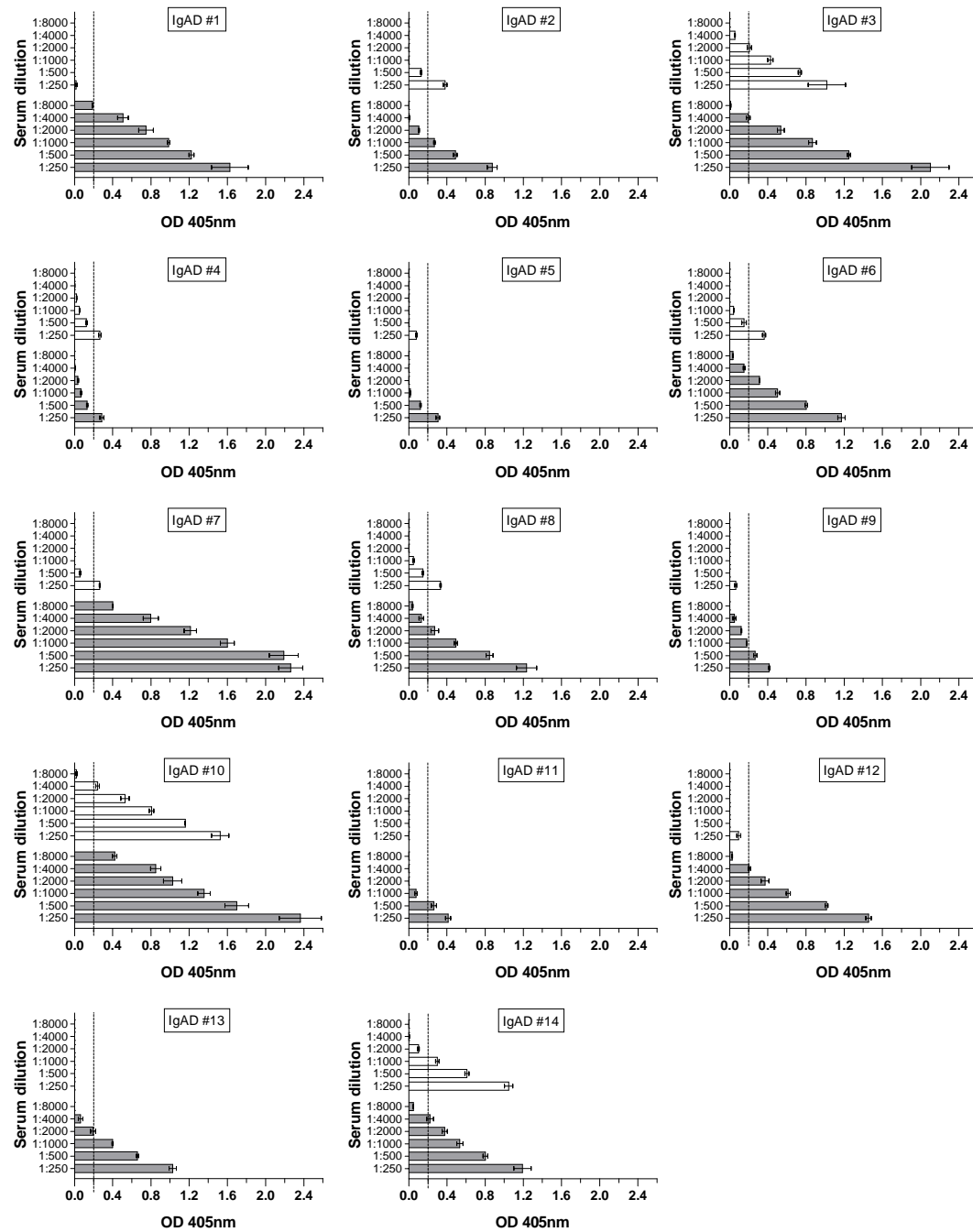
Figure 4. Detection of IgG anti-human and anti-bovine IgA antibodies in IgA-deficient sera. IgG anti-human IgA antibodies (clear bars) and IgG anti-bovine IgA antibodies (dark bars) were detected in serum by ELISA. Results are shown 20 mins after addition of substrate. Values (OD_{405nm}) are given as means ± SD. Means of background signals (1:250 dilution of serum reacting with coating buffer only) were deducted from the mean experimental values. The raw mean background values for all IgA-deficient sera ranged from 0.011-0.262. Cutoff for negative antibody titre is shown by a vertical dashed line.

The IgA-deficient sera are grouped in *Table 3* according to their titre of IgG anti-human IgA antibodies. Of the 14 IgA-deficient individuals tested, 8 (57%) were found to have IgG auto-antibodies against human IgA. These are IgAD samples #2, 3, 4, 6, 7, 8, 10 and 14. Sera that recorded a mean absorbance of less than 0.2 when tested with a serum dilution of 1:250 are considered negative for IgG anti-human IgA antibodies. Although all samples obtained from the American Red Cross were tested and found positive for anti-IgA antibodies at that facility, the results show that IgAD sera # 1, 5, 9, 11, 12 and 13 did not contain auto-reactive IgG anti-human IgA antibodies. These differences may be due to the method used for evaluating the presence of anti-IgA antibodies. The Red Cross used a passive haemagglutination assay whereas ELISA was used exclusively in this study. However, ELISA is a much more sensitive assay than haemagglutination. The most plausible explanation for the different results is the fact that in this study only IgG anti-IgA are scored whereas the haemagglutination method used by the Red Cross laboratory most probably scored anti-IgA antibodies of all isotypes. In this regard, it is important to note that a low level of IgM anti-IgA antibodies can be found in most people. These antibodies, which constitute most of the so called 'natural antibodies', are not associated with any pathology and are probably a mixture of antibodies specific to various Ig superfamily-related antigens (46;47).

On the other hand, all of the IgA-deficient samples (100%) were found to have IgG anti-bovine IgA antibodies. Of the 14 individuals tested, 10 (71%) had medium to high levels of IgG anti-bovine IgA antibodies. As seen in *Table 3*, response to bovine IgA is not linearly correlated with the response to human IgA. That is, some sera with low anti-human IgA antibodies have high levels of anti-bovine IgA antibodies. Such sera are IgAD #1, 12

and 13. In all IgA-deficient sera, the response to bovine IgA is stronger than the response to human IgA. This result strongly supports the notion that the initial antigenic trigger was bovine rather than human IgA.

TABLE 3: IgG anti-human and anti-bovine IgA antibody titres in IgA-deficient sera

IgAD sample number	IgG anti-huIgA titre	IgG anti-bovIgA titre	Level of IgG anti-bovIgA
# 10	1:4000	1:8000	High
# 3	1:2000	1:2000	Medium
# 14	1:1000	1:4000	High
# 2	1:250	1:1000	Medium
# 6	1:250	1:2000	Medium
# 8	1:250	1:2000	Medium
# 4	1:250	1:250	Low
# 7	1:250	1:8000	High
# 12	-	1:4000	High
# 5	-	1:250	Low
# 9	-	1:500	Low
# 1	-	1:4000	High
# 13	-	1:1000	High
# 11	-	1:500	Low

* Low titre 1:250 - 1:500, Medium titre 1:1000 - 1:2000, High titre 1:4000 - 1:8000.

IgG anti-bovine IgA antibodies are present in most control sera

Since anti-bovine IgA antibodies were found to be present independent of anti-human IgA antibodies, we hypothesized that antibody responses against bovine IgA are probably quite common and should be found in normal individuals. In that case, only a few of these individuals will develop anti-bovine IgA antibodies that will cross react with their own IgA, thereby leading to sIgAD. To test this, 18 control sera and one pooled human serum sample were tested for the presence of IgG anti-bovine and anti-human IgA antibodies. All of the control samples have normal levels of IgA (*Table 2*) and therefore by definition they do not

have any class specific anti-human IgA antibodies in their serum. ELISA was employed to look for the presence of IgG anti-bovine and anti-human IgA antibodies in the control serum samples. *Figure 5* shows the levels of IgG anti-human IgA (clear bars) and IgG anti-bovine IgA (dark bars) in control individuals. As previously mentioned, the graphs show the absorbance obtained at each dilution of serum (mean of triplicates). Antibody titres for each control serum are presented in *Table 4*. The antibody titre is defined here as the last serum dilution that recorded an absorbance (mean of triplicates) higher than 0.2. This cutoff is marked on each graph by a vertical dashed line.

As shown in *Figure 5* and *Table 4*, all control sera recorded a mean absorbance of less than 0.2 and are thus considered negative for IgG anti-human IgA antibodies. In contrast, out of the 18 control individuals tested, 11 (61%) had IgG anti-bovine IgA antibodies in their serum. Medium-titre antibodies were present in 3 of these sera, while the other 8 had low titre antibodies. Pooled human serum purchased from Sigma-Aldrich was also found to contain anti-bovine IgA antibodies, reflecting their significant presence in sera from normal individuals.
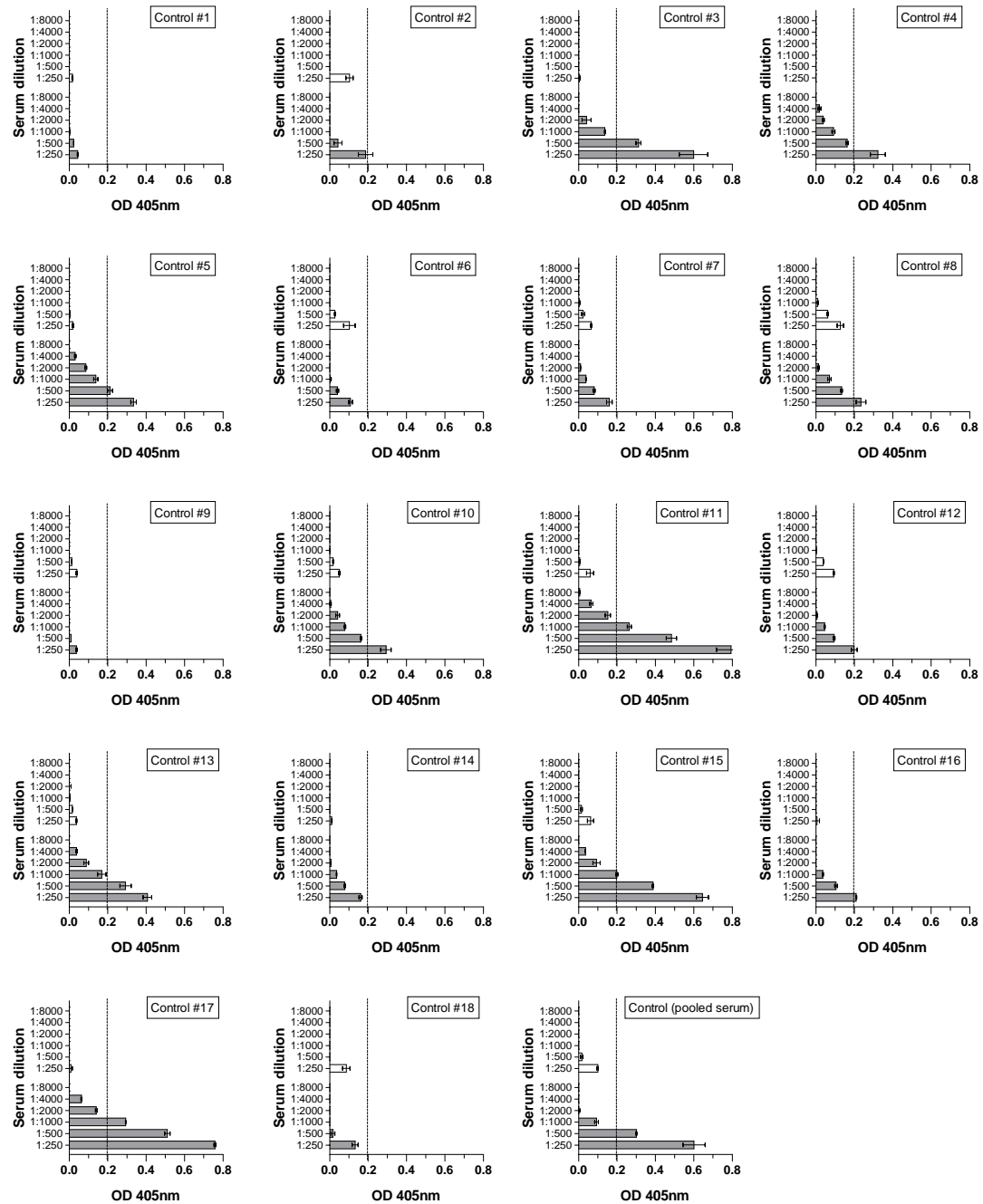
Figure 5. Detection of IgG anti-human and anti-bovine IgA antibodies in control sera. IgG anti-human IgA antibodies (clear bars) and IgG anti-bovine IgA antibodies (dark bars) were detected in serum by ELISA. Results are shown 20 mins after addition of substrate. Values (OD$_{405nm}$) are given as means ± SD. Means of background signals (1:250 dilution of serum reacting with coating buffer only) were deducted from the mean experimental values. The raw mean background values for all IgA-deficient sera ranged from 0.024-0.114. Cutoff for negative antibody titre is shown by a vertical dashed line.

TABLE 4: IgG anti-human and anti-bovine IgA antibody titres in control sera

Control sample no.	IgG anti-human IgA titre	IgG anti-bovine IgA titre
# 11	-	1:1000
# 17	-	1:1000
# 15	-	1:1000
# 3	-	1:500
# 13	-	1:500
# 5	-	1:500
# 4	-	1:250
# 10	-	1:250
# 8	-	1:250
# 16	-	1:250
# 12	-	1:250
# 2	-	-
# 14	-	-
# 7	-	-
# 18	-	-
# 6	-	-
# 1	-	-
# 9	-	-
Pooled human serum	-	1:500

The prevalence of anti-human IgA and anti-bovine IgA antibodies observed in all subjects is presented in *Table 5*.

TABLE 5: Prevalence of anti-human and anti-bovine IgA antibodies in IgA-deficient and control sera

	No. with IgG anti-human IgA antibodies	No. with IgG anti-bovine IgA antibodies
14 IgA-deficient individuals	8	14
18 control individuals	0	11

DISCUSSION

The existence of class specific anti-IgA antibodies in a significant percentage of IgA-deficient individuals has been known for almost 40 years, but their role in the etiology of sIgAD, if any, has never been established. Most investigators believe that the anti-human IgA antibodies found in IgA-deficient individuals are secondary to, and occur as a result of sIgAD, and therefore are not relevant to the etiology of the condition. The logic behind this claim is that IgA is a "foreign" protein in individuals with a complete absence of serum IgA and they are therefore likely to produce antibodies to this protein. However, there are several arguments against this being the case. In the aforementioned scenario, sIgAD always precedes the presence of anti-IgA antibodies. This would mean that all IgA deficiencies are congenital, but there is no evidence to support this. Moreover, in a long-term study by Koskinen *et al.*, only 2 of 127 IgA-deficient individuals without anti-IgA antibodies (1%) developed a high titre of these antibodies over a period of 19 years (36).

Based on the findings of the current study and the initial results presented to us by patient #14, we put forward the hypothesis that the class-specific anti-IgA antibodies found in 20-40% of people with sIgAD are actually the primary cause for the deficiency. The obvious question here is the identity and source of the immunizing agent that triggers the production of the anti-IgA antibodies in these individuals.

Several candidate sources are discussed in the literature. Although they all pertain to the theory that anti-IgA antibodies are secondary to the lack of IgA, they are equally relevant as potential antigenic sources. The most obvious one is human IgA and several exposure routes were suggested. The first is exposure to human IgA from blood products

(41). However, most studies find no correlation between previous exposure to IgA and the presence of anti-IgA antibodies (6;48;49). Other suggested routes of exposure include immunization of the mother during delivery via foetal bleeding into the maternal circulation (42), foetal exposure to maternal IgA via damaged placental villi *in utero* (42), sensitisation to colostral IgA while breast-feeding (37), and reaction to small but undetectable amounts of endogenous serum or cell surface IgA (37). However, none of these theories are supported by any evidence or statistics. For example, foetal or early post-natal exposure to maternal IgA would suggest that non-congenital IgA deficiency with verified presence of anti-IgA antibodies should appear very early in life, but there is no evidence for this. Sensitization to maternal human IgA when breast-feeding should result in a difference in incidence among breast-fed versus formula-fed infants, which has never been reported. Immunization of the mother during pregnancy is even less likely to cause induction of anti-IgA antibodies since it would imply that there should be a an increase in IgA deficiencies in women post-partum and this is not the case. It would also imply a skewed sex ratio in favour of females, but a difference in frequency among males and females has never been reported. A study of sIgAD and anti-IgA antibodies in 28,000 pregnant women revealed an incidence identical to that of the donor population of both sexes (50).

The other potential source of stimulatory antigen for the production of anti-IgA antibodies is xenogeneic IgA absorbed via the intestinal wall, with cows' milk being the only mentioned source for bovine IgA (29;51). There is only circumstantial support for this notion in the literature. For example, it was reported that a high incidence of antibodies to bovine milk proteins was found in the sera of IgA-deficient individuals (52;53). In these studies, the response was measured only against total milk and serum proteins so it is impossible to

ascertain if anti-IgA antibodies were part of this response. In later studies it was reported that the highest response against milk proteins was to the bovine gamma globulin (BGG) fraction which contains Igs of all isotypes (54). Since bovine milk contains only low amounts of IgA (roughly 0.25 mg/ml) (55), it is unclear what part of the response was against IgA.

As for our hypothesis, we strongly favour the possibility that the immunizing antigen is a xenogeneic IgA molecule, most likely bovine IgA. Furthermore, we predict that bovine IgA induces an immune response in a large proportion of the normal population, but only in some individuals these antibodies will cross-react with self/endogenous human IgA and lead to the removal of all self IgA, thereby resulting in sIgAD.

To test our hypothesis, the levels of IgG anti-human and anti-bovine IgA antibodies were determined in sera from both normal and IgA-deficient individuals. Anti-human IgA antibodies were found only in sera which also have anti-bovine IgA antibodies, but not vice versa. All 14 of the IgA-deficient individuals tested also had IgG anti-bovine IgA antibodies (100%), whereas only 8 had IgG anti-human IgA antibodies (57%). Of the 18 normal individuals tested, a surprisingly high proportion (61%) had IgG anti-bovine IgA antibodies in their serum, without anti-human IgA antibodies, and with normal levels of serum IgA. These results strongly support the hypothesis. Another interesting finding in support of the hypothesis is the fact that in all individuals with antibodies to both bovine and human IgA, the titre against bovine IgA is always higher (*Table 3*). This strongly supports the notion that the priming antigen is bovine IgA rather than human IgA. Another prediction that could be made is that in IgA-deficient individuals, the anti-bovine IgA titre would be much higher than that found in normal people with anti-bovine IgA antibodies which is exactly the case (*Table 3* versus *Table 4*). This is because in IgA-deficient individuals, the anti-bovine IgA

antibodies cross-react with self IgA and are therefore continually stimulated by the endogenous molecule. However, in normal people with anti-bovine IgA antibodies, bovine IgA is the only stimulating antigen and it is obviously present in much lower amounts and over shorter intervals.

Two obvious questions arise from our hypothesis: why is bovine IgA immunogenic in a large proportion of the population and why is the response to bovine IgA sometimes cross-reactive to human IgA. The issue of immunogenicity of food-derived antigens is complicated, controversial and beyond the scope of this thesis. However, it is generally accepted that most food-derived antigens do not elicit an immune response in most people. Are immunoglobulins in general, or IgA in particular, uniquely immunogenic? There are no direct studies on this subject but one could argue that ingested bovine secretory IgA (or other mammalian IgA for that matter) is preferentially uptaken by the gut epithelium M cells in Peyer's Patches since it can utilize the re-entry pathway of endogenous IgA (56). Interestingly, the ability of IgA to bind M cells is dependent on sequences found in the Cα1 and Cα2 domains of IgA and not in the secretory component. This binding is restricted to the IgA isotype so that M cells do not bind IgG and IgM. More importantly, adherence to M cells is species-independent which means that bovine IgA may bind to and be internalized by human M cells (57). It is meaningful that M cell binding is independent of secretory component, since it would entail that monomeric IgA, found mostly in serum, would bind as efficiently to M cells in the gut and would therefore be as immunogenic as dimeric secretory IgA, the predominant form of IgA found in milk. This could also explain the reduction in anti-IgA antibody levels in patient #14 after ceasing consumption of beef. It should be mentioned that cows' milk contains very low levels of IgA as compared to IgG

(58) and therefore beef would provide a higher source of bovine IgA than milk.

As for the second issue of cross-reactivity between bovine and human IgA, it is well-documented that cross-reactivity between the same antibody isotypes is very high among different species (59). In fact, there is a greater cross-reactivity between the same isotypes from different species than between different isotypes of the same species (i.e., human IgG is more likely to be recognized by antisera against bovine IgG, than by antisera against human IgA). It is therefore very plausible that bovine IgA is not unique in inducing cross-reactive antibodies to human IgA and in some cases, they may be induced by swine or sheep IgA. This will be tested in future experiments. The fact that most anti-bovine IgA antibodies raised in humans are not cross reactive (as evident from our finding that anti-bovine but not anti-human IgA antibodies can be detected in ~60% of normal people) is not surprising, given that bovine α-chain is quite different from human α-chain (only 68% identity) (60).

Finally, our hypothesis would predict that the acquired form of sIgAD, where anti-human IgA antibodies are present in serum, will not be found in people who are vegetarian from birth. Also, individuals from such populations should not have anti-bovine IgA antibodies in their serum. It would be interesting to confirm this in large vegetarian populations from countries such as India, where beef products are entirely absent from the average diet. Unfortunately, switching to a vegetarian or vegan diet is not expected to completely alleviate all of the symptoms and to eliminate the auto-antibodies entirely, owing to the continuous production of endogenous IgA. Such a change in diet may, however, lead to a long-term reduction in auto-antibodies, depending on the level of cross-reaction and their affinity to human IgA.

REFERENCES

1   Woof, J. M. and Kerr, M. A. 2006. The function of immunoglobulin A in immunity. *J. Pathol.* 208:270-282.

2   Hanson, L. A., Bjorkander, J., and Oxelius, V. A. 1983. Selective IgA deficiency. In Chandra, R. K., ed., *Primary and secondary immunodeficiency disorders*, pp. 62-81. Churchill Livingstone, Edinburgh.

3   Burrows, P. D. and Cooper, M. D. 1997. IgA deficiency. *Adv. Immunol.* 65:245-276.

4   Latiff, A. H. and Kerr, M. A. 2007. The clinical significance of immunoglobulin A deficiency. *Ann. Clin. Biochem.* 44:131-139.

5   Koistinen, J. 1975. Selective IgA deficiency in blood donors. *Vox Sang.* 29:192-202.

6   Vyas, G. N., Perkins, H. A., Yang, Y. M., and Basantani, G. K. 1975. Healthy blood donors with selective absence of immunoglobulin A: prevention of anaphylactic transfusion reactions caused by antibodies to IgA. *J. Lab Clin. Med.* 85:838-842.

7   Holt, P. D., Tandy, N. P., and Anstee, D. J. 1977. Screening of blood donors for IgA deficiency: a study of the donor population of south-west England. *J. Clin. Pathol.* 30:1007-1010.

8   Wells, J. V., McNally, M. P., and King, M. A. 1980. Selective IgA deficiency in Australian blood donors. *Aust. N. Z. J. Med.* 10:410-413.

9   Ropars, C., Muller, A., Paint, N., Beige, D., and Avenard, G. 1982. Large scale detection of IgA deficient blood donors. *J. Immunol. Methods* 54:183-189.

10   Clark, J. A., Callicoat, P. A., Brenner, N. A., Bradley, C. A., and Smith, D. M., Jr. 1983. Selective IgA deficiency in blood donors. *Am. J. Clin. Pathol.* 80:210-213.

11   Strothman, R., White, M. B., Testin, J., Chen, S. N., and Ball, M. J. 1986. HLA and IgA deficiency in blood donors. *Hum. Immunol.* 16:289-294.

12   Koskinen, S. 1996. Long-term follow-up of health in blood donors with primary selective IgA deficiency. *J. Clin. Immunol.* 16:165-170.

13   Kaneko, H., Kondo, N., Motoyoshi, F., Mori, S., Kobayashi, Y., Ozawa, T., and Orii, T. 1990. Expression of the alpha-chain gene in heterogeneous IgA immunodeficiency. *Scand. J. Immunol.* 32:483-489.

14   Islam, K. B., Baskin, B., Nilsson, L., Hammarstrom, L., Sideras, P., and Smith, C. I. 1994. Molecular analysis of IgA deficiency. Evidence for impaired switching to IgA. *J.*

Immunol. 152:1442-1452.

15   Asano, T., Kaneko, H., Terada, T., Kasahara, Y., Fukao, T., Kasahara, K., and Kondo, N. 2004. Molecular analysis of B-cell differentiation in selective or partial IgA deficiency. *Clin. Exp. Immunol.* 136:284-290.

16   Hummelshoj, L., Ryder, L. P., Nielsen, L. K., Nielsen, C. H., and Poulsen, L. K. 2006. Class switch recombination in selective IgA-deficient subjects. *Clin. Exp. Immunol.* 144:458-466.

17   Hammarstrom, L., Lonnqvist, B., Ringden, O., Smith, C. I., and Wiebe, T. 1985. Transfer of IgA deficiency to a bone-marrow-grafted patient with aplastic anaemia. *Lancet* 1:778-781.

18   Kanoh, T., Mizumoto, T., Yasuda, N., Koya, M., Ohno, Y., Uchino, H., Yoshimura, K., Ohkubo, Y., and Yamaguchi, H. 1986. Selective IgA deficiency in Japanese blood donors: frequency and statistical analysis. *Vox Sang.* 50:81-86.

19   Huntley, C. C. and Stephenson, R. L. 1968. IgA deficiency: family studies. *N. C. Med. J.* 29:325-331.

20   De Laat, P. C., Weemaes, C. M., Bakkeren, J. A., van den Brandt, F. C., van Lith, T. G., de, G. R., Van Munster, P. J., and Stoelinga, G. B. 1991. Familial selective IgA deficiency with circulating anti-IgA antibodies: a distinct group of patients? *Clin. Immunol. Immunopathol.* 58:92-101.

21   Vorechovsky, I., Zetterquist, H., Paganelli, R., Koskinen, S., Webster, A. D., Bjorkander, J., Smith, C. I., and Hammarstrom, L. 1995. Family and linkage study of selective IgA deficiency and common variable immunodeficiency. *Clin. Immunol. Immunopathol.* 77:185-192.

22   Strothman, R. A., Sedestrom, L. M., Ball, M. J., and Chen, S. N. 1989. HLA association of anti-IgA antibody production. *Tissue Antigens* 34:141-144.

23   Castigli, E., Wilson, S. A., Garibyan, L., Rachid, R., Bonilla, F., Schneider, L., and Geha, R. S. 2005. TACI is mutant in common variable immunodeficiency and IgA deficiency. *Nat. Genet.* 37:829-834.

24   Hong, R. and Ammann, A. J. 1972. Selective absence of IgA. Autoimmune phenomena and autoimmune disease. *Am. J. Pathol.* 69:491-498.

25   Fudenberg, H. H., Gold, E. R., Vyas, G. N., and Mackenzie, M. R. 1968. Human antibodies to human IgA globulins. *Immunochemistry.* 5:203-206.

26   Sandler, S. G., Eckrich, R., Malamut, D., and Mallory, D. 1994. Hemagglutination assays for the diagnosis and prevention of IgA anaphylactic transfusion reactions. *Blood* 84:2031-2035.

27   Laschinger, C., Shepherd, F. A., and Naylor, D. H. 1984. Anti-IgA-mediated transfusion reactions in Canada. *Can. Med. Assoc. J.* 130:141-144.

28   Thibault, L., Beausejour, A., de Grandmont, M. J., Long, A., Goldman, M., and Chevrier, M. C. 2006. Establishment of an immunoglobulin A-deficient blood donor registry with a simple in-house screening enzyme-linked immunosorbent assay. *Transfusion* 46:2115-2121.

29   Nadorp, J. H., Voss, M., Buys, W. C., Van Munster, P. J., van Tongeren, J. H., Aalberse, R. C., and van, L. E. 1973. The significance of the presence of anti-IgA antibodies in individuals with an IgA deficiency. *Eur. J. Clin. Invest* 3:317-323.

30   Hammarstrom, L., Persson, M. A., and Smith, C. I. 1983. Anti-IgA in selective IgA deficiency. In vitro effects and Ig subclass pattern of human anti-IgA. *Scand. J. Immunol.* 18:509-513.

31   Ammann, A. J. and Hong, R. 1971. Selective IgA deficiency: presentation of 30 cases and a review of the literature. *Medicine (Baltimore)* 50:223-236.

32   Ferreira, A., Garcia Rodriguez, M. C., Lopez-Trascasa, M., Pascual, S. D., and Fontan, G. 1988. Anti-IgA antibodies in selective IgA deficiency and in primary immunodeficient patients treated with gamma-globulin. *Clin. Immunol. Immunopathol.* 47:199-207.

33   Bjorkander, J., Hammarstrom, L., Smith, C. I., Buckley, R. H., Cunningham-Rundles, C., and Hanson, L. A. 1987. Immunoglobulin prophylaxis in patients with antibody deficiency syndromes and anti-IgA antibodies. *J. Clin. Immunol.* 7:8-15.

34   Munks, R., Booth, J. R., and Sokol, R. J. 1998. A comprehensive IgA service provided by a blood transfusion center. *Immunohematol.* 14:155-160.

35   Koistinen, J., Cardenas, R. M., and Fudenberg, H. H. 1977. Anti-IgA antibodies of limited specificity in healthy IgA deficient subjects. *J. Immunogenet.* 4:295-300.

36   Koskinen, S., Tolo, H., Hirvonen, M., and Koistinen, J. 1995. Long-term follow-up of anti-IgA antibodies in healthy IgA-deficient adults. *J. Clin. Immunol.* 15:194-198.

37   Petty, R. E., Palmer, N. R., Cassidy, J. T., Tubergen, D. G., and Sullivan, D. B. 1979. The association of autoimmune diseases and anti-IgA antibodies in patients with selective IgA deficiency. *Clin. Exp. Immunol.* 37:83-88.

38   Koistinen, J. and Sarna, S. 1975. Immunological abnormalities in the sera of IgA-deficient blood donors. *Vox Sang.* 29:203-213.

39   Pai, M. K., Davison, M., Bedritis, I., and Zipursky, A. 1974. Selective IgA deficiency in Rh-negative women. *Vox Sang.* 27:87-91.

40   Ammann, A. J. and Hong, R. 1971. Selective IgA deficiency and autoimmunity. *N. Engl. J. Med.* 284:985-986.

41   Vyas, G. N., Perkins, H. A., and Fudenberg, H. H. 1968. Anaphylactoid transfusion reactions associated with anti-IgA. *Lancet* 2:312-315.

42   Vyas, G. N. and Fudenberg, H. H. 1969. Isoimmune anti-IgA causing anaphylactoid transfusion reactions. *N. Engl. J. Med.* 280:1073-1074.

43   Vyas, G. N., Holmdahl, L., Perkins, H. A., and Fudenberg, H. H. 1969. Serologic specificity of hun anti-IgA and its significance in transfusion. *Blood* 34:573-581.

44   Homburger, H. A., Smith, J. R., Jacob, G. L., Laschinger, C., Naylor, D. H., and Pineda, A. A. 1981. Measurement of anti-IgA antibodies by a two-site immunoradiometric assay. *Transfusion* 21:38-44.

45   Harlow, E. and Lane, D. 1988. Storing and purifying antibodies. In *Antibodies a laboratory manual*, pp. 283-318. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

46   Vollmers, H. P. and Brandlein, S. 2006. Natural IgM antibodies: from parias to parvenus. *Histol. Histopathol.* 21:1355-1366.

47   Vollmers, H. P. and Brandlein, S. 2006. Natural IgM antibodies: the orphaned molecules in immune surveillance. *Adv. Drug Deliv. Rev.* 58:755-765.

48   Leikola, J., Koistinen, J., Lehtinen, M., and Virolainen, M. 1973. IgA-induced anaphylactic transfusion reactions: a report of four cases. *Blood* 42:111-119.

49   Laschinger, C. A. and Naylor, D. H. 1983. Anti-IgA antibodies and transfusion reactions in Canada. *Can. Med. Assoc. J.* 128:381-382.

50   Petty, R. E., Sherry, D. D., and Johannson, J. 1985. Anti-IgA antibodies in pregnancy. *N. Engl. J. Med.* 313:1620-1625.

51   Ammann, A. J. and Hong, R. 1971. Anti-antiserum antibody as a cause of double precipitin rings in immunoglobulin quantitation and its relation to milk precipitins. *J. Immunol.* 106:567-569.

52   Buckley, R. H. and Dees, S. C. 1969. Correlation of milk precipitins with IgA deficiency. *N. Engl. J. Med.* 281:465-469.

53   Cunningham-Rundles, C., Brandeis, W. E., Good, R. A., and Day, N. K. 1978. Milk precipitins, circulating immune complexes, and IgA deficiency. *Proc. Natl. Acad. Sci. U. S. A* 75:3387-3389.

54   Cunningham-Rundles, C., Brandeis, W. E., Good, R. A., and Day, N. K. 1979.

Bovine antigens and the formation of circulating immune complexes in selective immunoglobulin A deficiency. *J. Clin. Invest* 64:272-279.

55   Butler, J. E. 1971. Characteristics of bovine immunoglobulins and related molecules. Review of the bovine immunoglobulins. *J. Dairy Sci.* 54:1315-1316.

56   Corthesy, B. 2007. Roundtrip ticket for secretory IgA: role in mucosal homeostasis? *J. Immunol.* 178:27-32.

57   Mantis, N. J., Cheung, M. C., Chintalacharuvu, K. R., Rey, J., Corthesy, B., and Neutra, M. R. 2002. Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor. *J. Immunol.* 169:1844-1851.

58   Butler, J. E. 1979. Immunologic aspects of breast feeding, antiinfectious activity of breast milk. *Semin. Perinatol.* 3:255-270.

59   Nisonoff, A., Hopper, J. E., and Spring, S. B. 1975. Evolution of the Immunoglobulins. In *The antibody molecule* , pp. 265-312. Academic Press, New York.

60   Brown, W. R., Rabbani, H., Butler, J. E., and Hammarstrom, L. 1997. Characterization of the bovine C alpha gene. *Immunology* 91:1-6.