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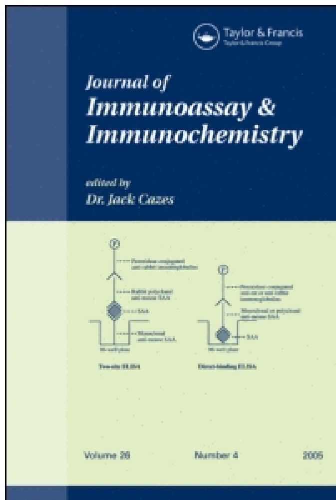

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
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High Prevalence of Human Anti-bovine IgG Antibodies as the Major Cause of False Positive Reactions in Two-Site Immunoassays Based on Monoclonal Antibodies

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Online publication date: 09 February 2004

To cite this Article Andersen, Ditte C. , Koch, Claus , Jensen, Charlotte H. , Skjødt, Karsten , Brandt, Jette and Teisner, Børge(2005) 'High Prevalence of Human Anti-bovine IgG Antibodies as the Major Cause of False Positive Reactions in Two-Site Immunoassays Based on Monoclonal Antibodies', Journal of Immunoassay and Immunochemistry, 25: 1, 17 — 30
To link to this Article: DOI: 10.1081/IAS-120027223
URL: http://dx.doi.org/10.1081/IAS-120027223

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JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 25, No. 1, pp. 17–30, 2004

High Prevalence of Human Anti-bovine IgG Antibodies as the Major Cause of False Positive Reactions in Two-Site Immunoassays Based on Monoclonal Antibodies

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ABSTRACT

A sandwich ELISA for quantification of the endometrial protein PP14 revealed false positive reactions in 81% of male sera ($n = 54$). The PP14 ELISA was based on two monoclonal antibodies (Mabs) with different epitope specificities—a catcher and a biotinylated indicator. The monoclonal antibodies were purified by protein G affinity chromatography from culture supernatant containing 10% (v/v) fetal calf serum (FCS). Human anti-animal IgG (bovine, mouse, horse, and swine)

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DOI: 10.1081/IAS-120027223
1532-1819 (Print); 1532-4230 (Online)
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antibodies and human anti-bovine serum albumin antibodies were measured using an ELISA design, with direct bridging of the solid phase and biotinylated antigens. The false positive reactions were abolished by addition of 1% (v/v) bovine serum to the dilution buffer (DB). Human anti-bovine IgG antibodies (HABIA) were detected in 99 out of 104 sera from blood donors (50 females; 54 males). HABIA levels in male sera ($n = 54$) were positively correlated to the false positive signals in the PP14 ELISA ($r = 0.923$; $p < 0.0001$). Antibodies to IgG from other mammalian species (mouse, horse, and swine) were also detected in the donor sera, but levels and frequencies were lower compared to that of HABIA. Furthermore, HABIA were positively correlated to human anti-bovine serum albumin antibodies in the donor sera ($r = 0.639$; $p < 0.0001$; $n = 103$). HABIA (prevalence 95%) cause false positive reactions due to crossbinding of contaminating bovine IgG and/or crossreaction with mouse IgG in two-site immunoassays. The apparent presence of human anti-mouse IgG antibodies (HAMA), described to create false positive results, may be due to a crossreacting fraction of the polyclonal circulating antibodies against bovine IgG.

Key Words: ELISA; HAMA; HAAA; Monoclonal antibodies; Human anti-bovine IgG antibodies (HABIA).

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INTRODUCTION

Two-site immunoassays, i.e., ELISA techniques, are renowned for their high degree of specificity and low detection limit, making them very suitable for quantitative and qualitative detection of serum proteins. False positive results caused by cross-linking of capture and label antibody in the abscence of specific analyte is, however, a major problem. As reviewed by Kohse and Wisser,[1] heterophilic antibodies (HA) and human anti-animal antibodies (HAAA) present in the analyte (e.g., serum or plasma) seem to be the major causes for this "Bridging interference," but due to their relatively low titers, they have often been an unrecognized source of error. Identifying assay interference and its nature, is of great importance in validation of results that otherwise may lead to false conclusions.

In present and recent literature, there has been a great deal of confusion distinguishing between naturally occurring HA and HAAA, the latter being the result of immunization, coincidental or not. Sometimes, it is difficult to classify if the antibody is a HA or a HAAA, though Kaplan and Levinson[2] suggest that antibodies are classified as "true" HAs if (i) no history of medical treatment with animal immunoglobulins or animal handling exist and (ii) if multispecificity is observed (i.e., weak reactivity against immunoglobulins

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from at least two different species). Often there is strong avidity against immunoglobulin from one species compared to other species, and here, additional testing is required to confirm the etiology of the interfering substance.

Many cases of immunoassay interference due to human anti-mouse antibodies (HAMA) have been reported and reviewed,[3−5] and HAMA has been suggested to be the most common HAAA.[3] However, in the majority of the cited reports, assay interference is only reduced and not completely removed by the addition of nonimmune mouse serum. We believe that this partial assay improvement may be accounted for by poor elucidation of the interferent.

In the present study, false positive results in an ELISA specific for the endometrial protein PP14 were analyzed. Circulating levels of PP14 are considered as a marker of endometrial receptivity in women (in vitro fertilization) and PP14 is not detectable in male serum.[6] The PP14 ELISA is designed with two monoclonal antibodies (Mab) with different epitope specificities, one being immobilized as catcher and the other being used as biotinylated indicator. A panel of serum samples from 104 apparently healthy blood donors were analyzed for the presence of various heterologous immunoglobulins (bovine, mouse, swine, rabbit, and horse) and their relation to assay interference in the PP14 ELISA.

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EXPERIMENTAL

Serum Samples

Serum samples from 104 apparently healthy donors (54 females and 50 males; age: 21 to 57 years) were obtained from the blood bank, Department of Clinical Immunology, Odense University Hospital, Odense, Denmark.

Monoclonal Antibodies

Balb/c mice were immunized subcutaneously with (3 times, 14 days interval) 25 μg endometrial protein PP14[6] purified from 2nd trimester human amniotic fluid, followed by an intravenous boost 4 days prior to fusion. Fusion was performed according to Köhler and Milstein,[7] as modified by Reading,[8] using SP2/0-Ag14 as a fusion partner for the spleen cells and polyethylene glycol 1500 as fusogen. Cells from anti-PP14 positive wells were cloned and recloned 3 times using limiting dilution.[9]

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IgG Purification

Protein G affinity chromatography was performed according to the manufacturers recommendations (Amersham Biosciences, UK), using 5 mL HiTrap Protein G columns attached to an Äkta Fast Performance Liquid Chromatography system (Amersham Biosciences, UK). In order to avoid cross-contamination in purification of IgG from different species, new matrices were used for (i) hybridoma culture supernatants, (ii) bovine serum, (iii) mouse serum, (iv) horse serum, (v) swine serum, and (vi) rabbit serum.

ELISA Techniques

General Reagents and Standard Procedures

Maxisorp 96 wells microtiterplates (NUNC A/S, Roskilde, Denmark) were used throughout the study. Coating was performed overnight (o/n) at 4°C in 100 mmol/L carbonate buffer, pH 9.6. All incubations of 0–2 hrs duration were performed at room temperature while 4°C was used at o/n incubations.

Phosphate buffered saline (PBS) containing 0.05% (v/v) Tween 20 was used as the dilution buffer (DB) and washing buffer. In some experiments, the DB was supplemented with 1% (v/v) animal serum (bovine, mouse, or horse).

Washing was repeated four times between each assay step.

H_2O_2/orthophenyldiamine (OPD), 100 µL, was used as a substrate/hydrogen donor and the reaction was stopped by the addition of 100 µL 1 mol/L H_2SO_4 to each well. The plates were read at 492 nm.

Assays

Identification of anti-PP14 producing hybridomas was performed on microtiter plates coated with affinity purified rabbit anti-PP14 antibodies (1 µg/mL), followed by PP14 saturation using amniotic fluid (diluted 1 : 250, 2 hrs). A culture supernatant, 100 µL, was added (incubation 1 h), and the presence of mouse anti-PP14 antibodies was indicated by incubation (30 min.) with peroxidase labelled rabbit anti-mouse Ig (P260, DakoCytomation, Copenhagen, Denmark), diluted 1 : 1000. The same ELISA technique was used during cloning.

The quantitative PP14 ELISA was based on 2 Mabs (Mab 16 and Mab 18) with different epitope specificities. Mab 16 was used as catcher and biotinylated Mab 18 as indicator antibody (incubation 1 h). A dilution series of 2nd trimester amniotic fluid was used as calibrator, and human serum samples

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($n = 104$) were applied in dilution series, starting 1 : 3 and incubated o/n. The assay was developed using streptavidin-peroxidase (PO397, DakoCytomation, Copenhagen, Denmark), diluted 1 : 3000 (incubation 30 min.) followed by H_2O_2/OPD.

Analysis of human anti-animal Ig antibodies was performed on microtiterplates coated with protein G purified animal (bovine, mouse, horse, swine, or rabbit) IgG (1 μg/mL). The coated plates were incubated (o/n) with a dilution series (starting 1 : 3) of human sera ($n = 104$). Biotin labelled animal IgG, 100 μL, was added (diluted 1 : 1000; 2 hrs), followed by peroxidase-labelled streptavidin (30 min). Plates were developed with H_2O_2/OPD.

Analysis of human anti-bovine serum albumin (BSA) antibodies was performed on microtiterplates coated with BSA (1 μg/mL, Bovine albumin, SIGMA). The coated plates were incubated (o/n) with a dilution series (starting 1 : 3) of human sera ($n = 104$). Biotin labelled BSA, 100 μL, was added (1 : 2000, 2 hrs), followed by peroxidase-labelled streptavidin (30 min). Plates were developed with H_2O_2/OPD.

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Absorption of Human Anti-animal IgG Antibodies

The absorption experiments were performed by comparison of the ELISA signal in an anti-animal IgG assay using the normal DB and a buffer supplemented with 1% (v/v) animal serum. The inhibition was calculated as follows:

$$\frac{(X_{DB} - B_{DB}) - (X_{DB+} - B_{DB+})}{X_{DB} - B_{DB}} \times 100 = \%\text{inhibition}$$

where X_{DB} and X_{DB+} are the signals in serum diluted in ordinary DB and DB with 1% animal serum (DB+), respectively. B_{DB} and B_{DB+} are means ($n = 20$) of serum blanks in the two buffers.

RESULTS

Antibodies and ELISA

The specificity of the monoclonal anti-PP14 antibodies was tested in ELISA (see Experimental) and further confirmed by immunohistochemical analysis with the staining reaction restricted to the glandular cells of the endometrium.

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Following cloning, the hybridoma cells were expanded in culture medium containing 10% fetal calf serum (FCS), and the IgG was purified by protein G affinity chromatography.

The assay for PP14 quantification was established using Mab 16 as catcher- and biotinylated Mab 18 as indicator antibody. Mab 16 and Mab 18 reacted with independent epitopes on the PP14 molecule, and a dilution series of second trimester amniotic fluid was used as the calibrator in this assay.

False Positive Reaction and Preliminary Absorption

PP14 is synthesized by the glandular epithelium of the endometrium and PP14 is not detectable in serum from males, as previously demonstrated using an ELISA technique based on immunospecifically purified rabbit anti-PP14 antibodies.[6]

However, as seen from Fig. 1, false positive reactions were observed in 44 out of 54 male sera. The false positive reactions were abolished replacing the monoclonal catcher antibody (Mab 16) with affinity purified polyclonal rabbit anti-PP14 antibody. These observations made us speculate if the false positive reactions were due to the presence in male sera of HAMA, which crossbind the monoclonal catcher- and biotinylated indicator antibodies in the absence of PP14. However, the addition of 1% (v/v) mouse serum to the DB had only a

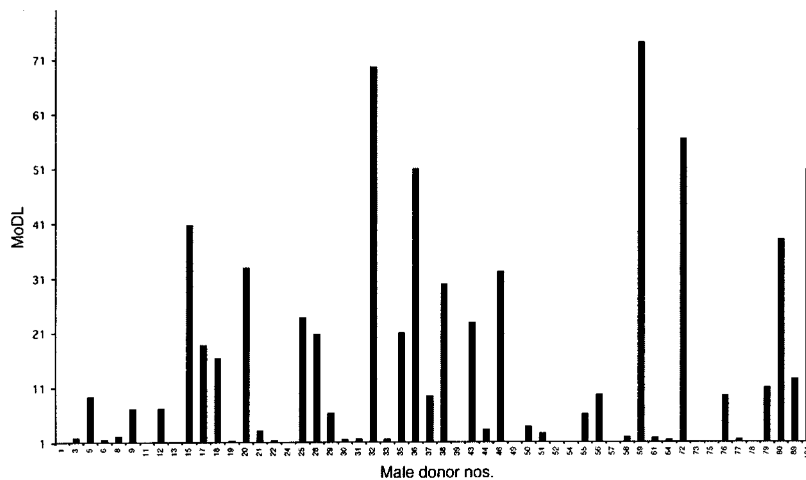

Figure 1. The signals obtained when 54 male sera were analyzed in the PP14 ELISA based on Mab. The signals are expressed as MoDL, the detection limit being defined as the mean ($n = 20$) of serum blank + 3SD.

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marginal effect in reducing the false positive reactions. By contrast, DB supplemented with 1% (v/v) FCS (intended to serve as control) tended to abolish all false positive reactions. These results suggested that the false positive reactions in the PP14 ELISA were due to human anti-bovine IgG antibodies (HABIA) rather than HAMA in the male sera. Thus, the hypothesis was: (i) bovine IgG originating from FCS was co-purified with the monoclonals on Protein G affinity chromatography, (ii) the microtiter plate was coated with a mixture of mouse IgG (Mab 16) and bovine IgG, (iii) the indicator preparation was a mixture of biotinylated mouse IgG (Mab 18) and biotinylated bovine IgG, and (iv) in the absence of PP14 (male serum) solid phase bovine IgG and biotinylated bovine IgG was crossbound by the HABIA present in human serum.

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Human Anti-bovine IgG-, Human Anti-BSA Antibodies, and the False Positive Reactions

The ELISA technique for analysis of HABIA antibodies was designed to mimic the cause of the false positive reactions described in the hypothesis above, i.e.,: (i) Maxisorp plates coated with purified bovine IgG, (ii) addition of human serum, and (iii) biotinylated bovine IgG. As seen from Fig. 2, HABIA was detected in 99 out of 104 donors, and no apparent difference was observed between men and women. The results are given as multiples of the detection limits (MoDL), the detection limit being defined as the mean of serum blank ($n = 20$) + 3SD. Figure 3 shows the positive and significant ($r = 0.923$; $p < 0.0001$; $n = 54$) correlation between serum levels of HABIA and the false positive reactions in the PP14 ELISA. Moreover, the false positive signals were completely abolished by addition of 1% bovine serum to the DB.

Human anti-BSA antibodies were analyzed in an ELISA design identical to that described for HABIA. The correlation between human anti-BSA antibodies and HABIA observed in 103 blood donors, is shown in Fig. 4. The positive correlation ($r = 0.639$; $p < 0.0001$; $n = 103$) suggests that the antibodies against bovine serum proteins is due to an active immunization process.

Human Antibodies Against Mouse IgG and Their Connection to Human Anti-bovine IgG Antibodies

Whereas, HABIA were detectable in 95.2% of the donors, antibodies against mouse IgG (i.e., HAMA) were only detected in 41.4% of the same cohorte ($n = 103$). The mean values of the multiples of detection limit

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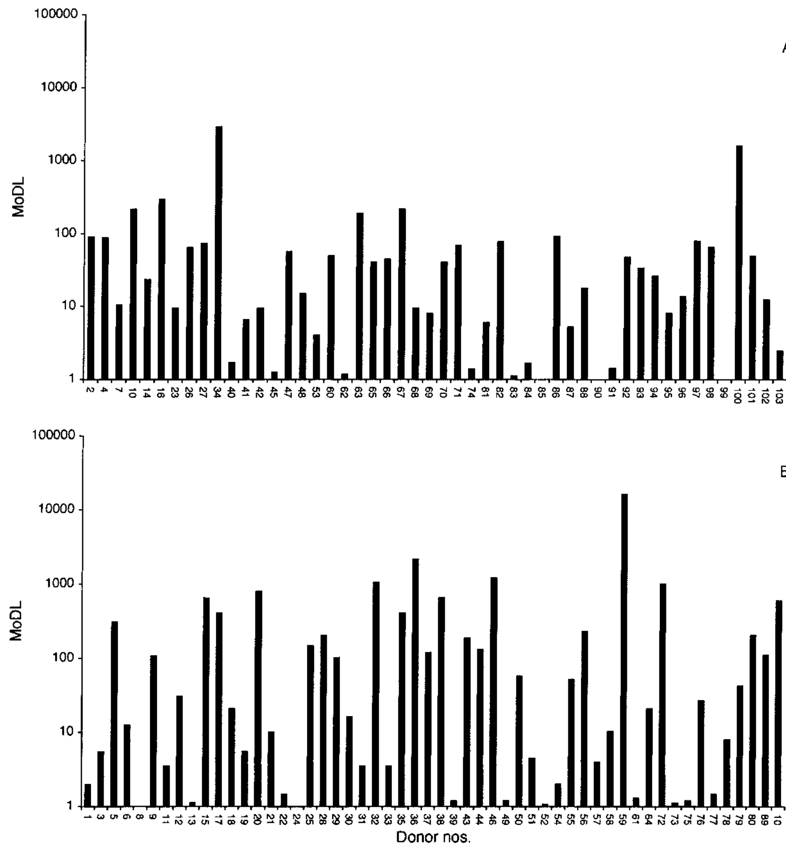

Figure 2. Measurements of HABIA levels in sera from (A) 50 female donors and (B) 54 male donors. The ELISA method was designed to mimic the ability of HABIA to crossbind the catcher- and biotinylated indicator antibodies. Values are expressed as MoDL.

(MoDL) based on log transformed data were 22.9 and 1.94 for HABIA and HAMA, respectively. The HABIA titers were positively correlated to HAMA ($r = 0.532$; $p < 0.0001$; $n = 103$).

As seen from Fig. 5A, the signal in the HABIA ELISA was completely abolished by the addition of 1% bovine serum to the DB, whereas, addition of 1% mouse serum only had a marginal effect (donor no. 90 had no detectable HABIA). The same absorption experiment was performed in the HAMA ELISA, and the results are shown in Fig. 5B. It is seen, that 1% bovine serum eliminated the signal in 11 out of 13 donors, as effective as 1% mouse serum, the sole exceptions being donor no. 27 and 90.

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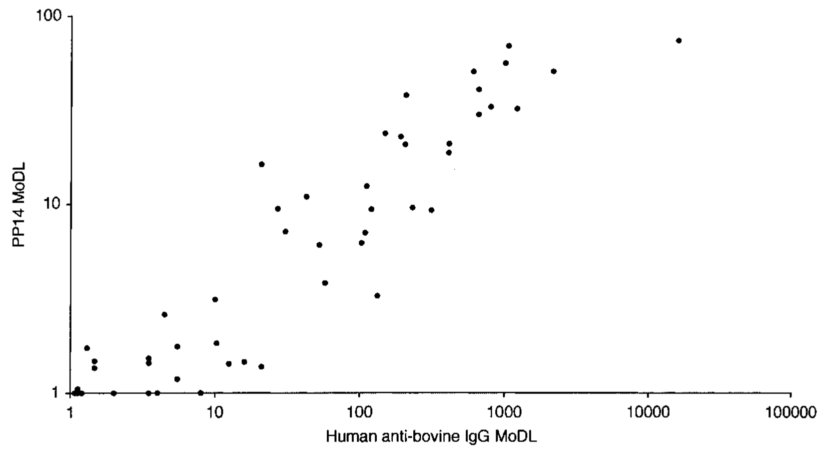

Figure 3. Correlation between false positive PP14 signals and the amount of HABIA in 54 males using the *Z*-correlation test on log transformed MoDL values ($r = 0.923$; $p < 0.0001$).

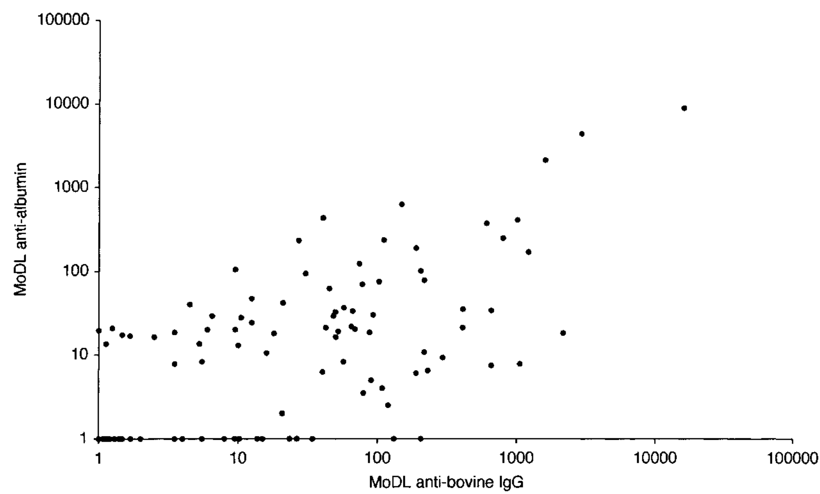

Figure 4. Correlation between HABIA and human anti-BSA antibodies in sera from 103 healthy subjects using the *Z*-correlation test on log transformed MoDL values ($r = 0.639$; $p < 0.0001$).

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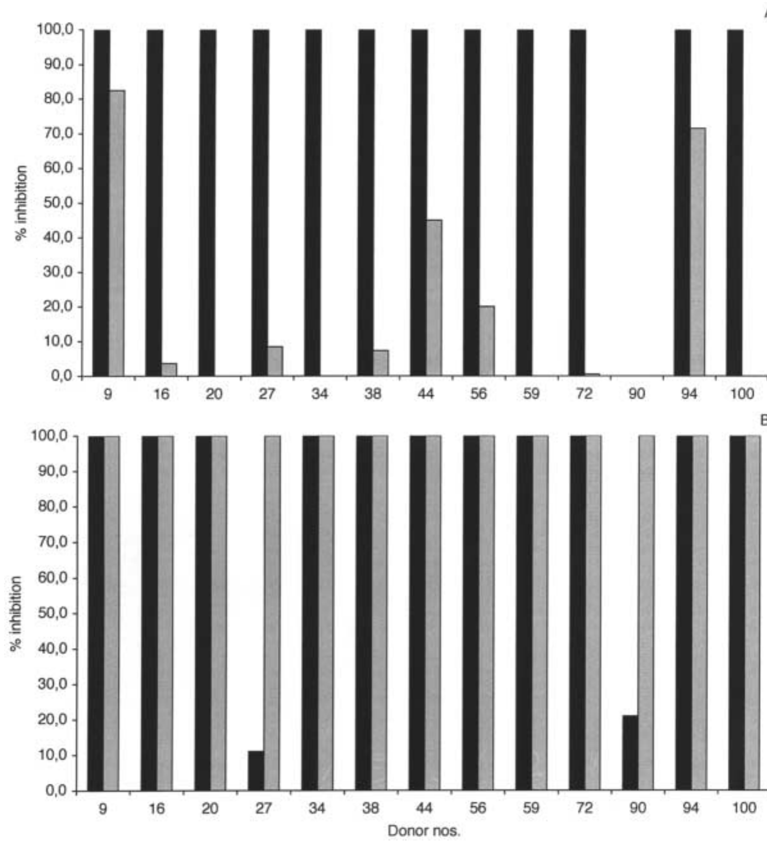

Figure 5. Analysis of HABIA (A) and human anti-mouse IgG antibodies (B) in sera from 13 selected donors. The analysis were performed with 1% bovine serum (■) or 1% mouse serum (■) in the DBs, and the inhibition calculated as described in Materials and Methods.

Likewise, the signal in the human anti-horse IgG antibody ELISA was efficiently removed by the addition of 1% bovine serum to the DB (data not shown).

DISCUSSION

The analyte, presented here to illustrate the effect of the interfering antibodies in a two-site immunoassay, is a gender specific protein, PP14,

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specifically synthesized by the glandular cells of the endometrium.[6,10] The ELISA technique for quantification of circulating PP14 was based on two Mabs with distinct epitope specificities, one used as catcher and the other as biotinylated indicator. False positive results in ELISA techniques based on mouse Mab have previously been reported for other analytes, as described in several reviews.[3,4,11]

Our initial PP14 assay showed significant signals in 81% of sera obtained from adult males, and these results initiated the hypothesis that the false positive signals were caused by the presence of HA or specific HAAA; the latter including either antibodies to mouse IgG (the specific Mab) or to bovine IgG originating from the fetal calf serum used in the culture medium for antibody producing hybridomas. The fact that the false positive reactions were only efficiently abolished by the addition of 1% (v/v) bovine serum to the DB, suggests that HABIA were the major confounding principle.

Antibodies in human sera with specificity against immunoglobulins from other species can be divided into 3 groups, i.e., rheumatoid factors, iatrogenic, and noniatrogenic anti-Ig antibodies. Due to the heterologous reactivity, such antibodies may interfere with two-site immunoassays, causing false positive reactions due to crossbinding of capture and detection antibodies, as they may diminish the signal by blocking analyte binding to capture and/or detection antibody.[3,4,11,12]

Iatrogenic antibodies to heterologous immunoglobulins are seen as a result of treatment with heterologous immunoglobulin preparations, most often mouse Mabs. Such treatment (although often accompanied with immunosuppressive therapy) may often lead to very high titres of e.g., HAMA, which can seriously interfere with two-site immunoassays.[3,4] However, the most challenging situation is when there is no obvious reason for the presence of antibodies to heterologous immunoglobulins. In our study, we observed that 95% of 104 healthy blood donors had significant levels of antibodies to bovine immunoglobulin (defined as signals higher than mean of blank + 3SD). The methods used in the present study to analyze for human anti-animal antibodies in serum was designed to mimic the false positive reaction in the test ELISA system (PP14 ELISA), i.e., the ability of the human antibodies to crossbind the coated and the biotinylated fluid phase Ig directly.

False positive PP14 results were obvious in 81% of the analyzed male sera ($n = 54$), and this false positive reaction was completely abolished by the addition of 1% bovine serum to the DB, whereas, addition of 1% mouse serum only had marginal effect. Moreover, there was a highly significant correlation between the false positive PP14 signal in males and the serum concentration of HABIA.

When the human sera ($n = 103$) were analyzed for antibodies against IgG from other species (mouse, horse, swine, and rabbit) levels and frequencies of

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positive results were substantially lower than those observed for HABIA. However, donor no. 90 was the sole exception since this donor had no HABIA, whereas, HAMA was positive at a low titer. The absorption experiments indicated that donor no. 90 (and perhaps donor no. 27) had antibodies against mouse IgG, which were not simply a result of HABIA crossreacting with mouse IgG.

These data suggest that the primary mechanism for the false positive signals is contamination of monoclonal antibody preparations with bovine Ig originating from the FCS. Such bovine IgG will be co-purified during protein-G affinity chromatography of the Mab and appear in the preparation of capture, as well as, indicator antibodies. False positive reactions are caused by direct bridging of solid phase bovine IgG and the biotinylated bovine IgG by HABIA in the absence of analyte. However, the problem is not solved by removal of contaminating bovine IgG from the antibody preparations, since the HABIA crossreact with mouse IgG. This also indicates that HABIA reacting with mouse IgG can easily be confused with HAMA.

The data presented here, strongly indicate HABIA as the major problem in two-site immunoassays based on Mab, and this is in agreement with the results published previously,[13–16] demonstrating bovine IgG to be superior to mouse IgG in the attempt to avoid false positive reactions. What is the origin of these HABIA? The significant positive correlation between HABIA and human anti-bovine serum albumin antibodies suggests that we are dealing with immunoantibodies rather than HA, but the immune response is not likely to be initiated by meat ingestion since the frequency and titer of human anti-swine IgG antibodies were much lower in the danish population compared to HABIA. Thus, we believe that cows milk and products of cows milk may be obvious candidates, although residual bovine proteins in vaccines used in childrens vaccination programmes offer an alternative explanation.

We conclude that HABIA and their crossreactions to IgG from other species, e.g., mouse IgG, have major implications in two-site immunoassays based on mouse Mab. The false positive reactions can be eliminated by application of catcher and detector antibodies from different species, or by the addition of bovine serum to the assay system, resulting in fluid phase absorption of the human anti-bovine reactivity.

ACKNOWLEDGMENTS

This work was supported by the Danish Medical Research Council, Arvid Nilssons Fond, Else og Aage Gronbeck-Olsens Fond, and Sigvald og Edith Rasmussens Legat.

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Received May 10, 2003
Accepted May 31, 2003
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