*Clinical Chemistry* 45:7
942–956 (1999)

Review

# Human Anti-Animal Antibody Interferences in Immunological Assays

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***Purpose:*** **The scope and significance of human anti-animal antibody interference in immunological assays is reviewed with an emphasis on human anti-animal immunoglobulins, particularly human anti-mouse antibodies (HAMAs).**

***Issues:*** **Anti-animal antibodies (IgG, IgA, IgM, IgE class, anti-isotype, and anti-idiotype specificity) arise as a result of iatrogenic and noniatrogenic causes and include human anti-mouse, -rabbit, -goat, -sheep, -cow, -pig, -rat, and -horse antibodies and antibodies with mixed specificity. Circulating antibodies can reach gram per liter concentrations and may persist for years. Prevalence estimates for anti-animal antibodies in the general population vary widely and range from <1% to 80%. Human anti-animal antibodies cause interferences in immunological assays. The most common human anti-animal antibody interferent is HAMA, which causes both positive and negative interferences in two-site mouse monoclonal antibody-based assays. Strategies to prevent the development of human anti-animal antibody responses include immunosuppressant therapy and the use of humanized, polyethylene glycolylated, or Fab fragments of antibody agents. Sample pretreatment or assay redesign can eliminate immunoassay interferences caused by anti-animal antibodies. Enzyme immunoassays, immunoradiometric assays, immunofluorescence, and HPLC assays have been designed to detect HAMA and other anti-animal antibodies, but intermethod comparability is complicated by differences in assay specificity and lack of standardization.**

***Conclusions:*** **Human anti-animal antibodies often go unnoticed, to the detriment of patient care. A heightened awareness on the part of laboratory staff and clinicians of the problems caused by this type of interference in routine immunoassay tests is desirable. Efforts should be directed at improving methods for identifying and eliminating this type of analytical interference.**



Circulating human antibodies reactive with animal proteins (anti-animal antibodies) are an often unrecognized and unsuspected source of interference in immunological assays, in particular two-site (sandwich) immunoassays. Although many human anti-animal antibodies may be detectable, the laboratory is mostly concerned with antibodies of sufficient titer and affinity to have an analytically significant effect. This type of assay interference was first noted for immunodiffusion assays *(1)*, but subsequently has been reported in a range of immunological assays *(2–8)*. In some cases, anti-animal antibodies arise as result of exposure to a defined antigen (e.g., mouse monoclonal antibody therapeutic agent), but in other cases, the antigens that gave rise to the anti-animal antibodies are ill-defined *(3)*. These antibodies include antibodies against animal immunoglobulins [e.g., human anti-mouse antibodies (HAMAs)[1] *(4, 5, 8, 9)*], animal albumins *(10)*, and insect glycoproteins *(11)*. Anti-animal antibodies are to be distinguished from the lower affinity heterophile antibodies, a term originally used to describe IgM antibodies associated with mononucleosis that agglutinated sheep red cells *(3)*, that have broader reactivity, e.g., antibodies against red cell proteins of different species (rat, sheep, horse, rabbit, guinea pig, and cow) *(12–18)*, such as Paul Bunnell antibody (a sheep erythrocyte agglutinin that is also reactive with horse, bovine, and goat erythrocytes).

The Food and Drug Administration (FDA) has recognized the importance of anti-animal antibodies such as HAMA. In its "review criteria for assessment" documents, the FDA recommends that the labeling (e.g., pack-

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Received January 19, 1999; accepted April 15, 1999.

[1] Nonstandard abbreviations: HAMA, human anti-mouse antibody; FDA, Food and Drug Administration; hCG, human chorionic gonadotropin; HARA, human anti-rabbit antibody; FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone; CK, creatine kinase; CEA, carcinoembryonic antigen; PEG, polyethylene glycol; IIR, Immunoglobulin Inhibiting Reagent; and HBR, Heterophilic Blocking Reagent.

age insert) of an in vitro diagnostic device list as a limitation the following: "As with any assay employing mouse antibodies, the possibility exists for interference by human anti-mouse antibodies (HAMA) in the sample" *(19)*. In more recent documents, the FDA recommends the following: " If the assay kit employs mouse monoclonal antibodies, include a warning that specimens from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibodies (HAMA) and may show either falsely elevated or depressed values when tested" *(20)*.

This review surveys the scope and extent of human anti-animal antibody interferences, and examines methods to eliminate formation of these antibodies and reagents and sample pretreatment protocols designed to combat analytical problems attributable to their presence in biological fluids. It focuses on human antibodies reactive with animal immunoglobulins (e.g., mouse and goat), and the reader is referred to previous reviews for additional information on this general topic *(2, 3, 8, 21, 22)*.

## Etiology of Anti-Animal Antibodies and Mechanism of Interference in Immunoassays

Circulating anti-animal antibodies can arise from iatrogenic and noniatrogenic causes. The former is the result of the normal response of the human immune system to an administered "foreign" protein antigen. Currently available diagnostic and pharmaceutical agents derived from an animal source are extensive and range from rodent immunoglobulins to hormones isolated from fish (Table 1) *(23–34)*. In addition, some recombinant proteins are affinity purified on immobilized monoclonal mouse antibody columns, and the possibility exists for some of the mouse monoclonal antibody to detach and copurify with the protein *(35)*.

Blood transfusion is also associated with an increased incidence of anti-animal antibodies. A study of 2829 participants in a population health survey revealed that 14.4% of the participants who had been transfused were anti-animal positive, compared with 10.4% of the participants who had never received a blood transfusion. This difference was presumably attributable to infusion of preexisting human anti-animal antibody or as a result of infusion of a foreign antigen present in the unit of blood *(36)*.

Vaccination against infectious diseases is another route by which animal protein antigens may be inadvertently presented to the immune system and trigger antibody formation. In the US, chick embryo or egg cultures are frequently used in vaccine production, and residual chicken protein may be present in vaccines, whereas in Europe, some vaccines contain rabbit serum, e.g., rubella vaccine in France, and multimicrobial vaccine (Bruschettini vaccine) in Italy *(32, 34)*.

The administration of unconventional therapies is also a route to immunization with animal protein. For example, a patient developed anti-rabbit antibodies following injections of "antireticulocytoxique", which is a lyophilized serum obtained from rabbits injected with homogenates of human bone marrow and spleen and is intended as a tonic to improve senescence and to reduce fatigue and debilitation *(34)*.

Noniatrogenic causes of anti-animal antibodies include maternal transfer across the placenta to the unborn child *(37, 38)*, animal husbandry or the keeping of animals as pets *(39)*, and the transfer of dietary antigens across the gut wall in conditions such as celiac disease *(40, 41)*. Anti-animal antibodies are also more common in multiparous females *(36)*, and a high incidence of human anti-animal antibodies has also been observed in association with certain disease states, e.g., idiopathic cardiomyopathy *(42)*.

**Table 1. Animal-derived pharmaceuticals.**

| Drug | Source | Ref. |
|---|---|---|
| Antibody-targeted imaging reagents | Mouse | 23 |
| | Rat | 24 |
| Antibody-targeted drugs | Mouse | 23 |
| | Rat | 24 |
| Anti-thymocyte globulin | Horse | 25 |
| | Rabbit | 26 |
| Anti-snake venom | Horse | 27 |
| Calcitonin | Salmon | 28 |
| Digibind (anti-digoxin Fab) | Sheep | 29 |
| Factor VIII | Pig | 30 |
| Insulin | Pig | 31 |
| Vaccines | Rabbit | 32 |
| | Chicken | 33 |
| Patent medicines | Rabbit | 34 |

## HAMA

HAMA is probably the most common type of human anti-animal antibody. The main cause and reason for the increase in the incidence of HAMA is the use of mouse monoclonal antibodies for therapeutic and imaging purposes (intraperitoneal, intravenous, and subcutaneous routes of administration in microgram to milligram doses) *(29, 43–49)*.

A mouse monoclonal antibody is a foreign protein, and in vivo it can trigger an immune response to produce HAMAs. Consequently, it is not surprising to find that in the vast majority of clinical trials with mouse monoclonal antibodies, many of patients were found to have developed a HAMA response following administration of the antibody (Table 2) *(50–74)*. The specificity of a monoclonal antibody permits targeting of a particular cell type or tissue. For example, monoclonal anti-OKT3 is widely used in transplantation as an immunosuppressant because it binds to the CD3 surface antigen on T lymphocytes and interferes with the ability of the cell to recognize foreign antigens. A further refinement is to attach drugs, toxins, or imaging agents to a monoclonal antibody and to use the resulting conjugates for the targeted delivery of these agents in high concentration to specific sites in the

**Table 2. Anti-animal response to monoclonal antibodies.**

| Monoclonal | Specificity | Condition | No. of patients developing antibodies (dose) | Ref. |
|---|---|---|---|---|
| Mouse | | | | |
| B-E8 | Interleukin-6 | Metastatic renal cell carcinoma | 9 of 12 | *50* |
| OKT3 | CD3 | Organ transplantation | 695 of 12 133 | *51* |
| | | Cardiac allograft | 8 of 55 | *52* |
| | | Cardiac transplant | 6 of 20 | *53* |
| B4 | CD19 | B-cell malignancy | 9 of 25 | *54* |
| BW 4 | Platelet | Thrombosis | 0 of 4 | *55* |
| BW 250/183 | Granulocyte | Inflammation | 1 of 20 | *56* |
| BW 431/26 | CEA | Colorectal carcinoma | 29% of 141 | *57* |
| BW 494 | Pancreatic carcinoma-associated glycoprotein | Pancreatic ductal carcinoma | 150 of 150 | *58* |
| | | Pancreatic carcinoma | 8 of 8 | *59* |
| CCR 086 | Mucin | Colorectal carcinoma | 4 of 5 (20 mg), 0 of 5 (5 mg) | *60* |
| CD21 AND CD24 | CD21, CD24 | Epstein-Barr virus-induced lymphoproliferative syndrome | 0 of 1 | *61* |
| EMD 55,900 | Epidermal growth factor receptor | Malignant gliomas | 1 of 16 | *62* |
| IMMU-4 | CEA | Colon and rectum carcinoma | 2 of 210 | *63* |
| LL2 | B cells | Non-Hodgkin lymphoma | 3 of 8 | *64* |
| Lym-1 | B cells | B-cell malignancy | 2 of 10 | *65* |
| MN-14 | CEA | CEA-producing tumors | 9 of 18 | *66* |
| NP-4 | CEA | Small volume tumors | 5 of 6 | *66* |
| NR-M1-05 | Melanoma antigen | Malignant melanoma | 69% of 20 | *67* |
| OKB7 | B cells | Non-Hodgkin lymphoma | 5 of 18 | *68* |
| XMMEN-0E5 | Bacterial endotoxin lipid A | Bacteremia | 3 of 9 | *69* |
| 13G2a | GD-2 | Neuroectodermal tumors | 16 of 18 | *70* |
| 30.6 | Anti-colon cancer | Colorectal carcinoma | 10 of 10 | *71* |
| 96.5 | p97 and 48.7 proteoglycan melanoma antigen | Melanoma | 4 of 5 | *72* |
| Rat | | | | |
| YTH 24.5 | CD45 | Renal | 2 of 40 | *73* |
| 33B3.1 | CD25 | Bone marrow transplant | 0 of 15 | *74* |

body (e.g., tumor tissue). Other proposed applications of mouse monoclonal antibodies include the use of antibodies with enzyme activity ("abzymes") as antiviral, anticancer, and thrombolytic therapeutic agents *(75)*.

### ANTIBODY TYPE AND SPECIFICITY

Human anti-animal antibody responses can be of the IgG, IgA, IgM, or rarely, the IgE class *(76–79)*. In the case of anti-animal antibodies elicited by animal immunoglobulins, the human anti-animal antibody can have anti-idiotype or anti-isotype specificity. Anti-idiotype antibodies are directed against the hypervariable region of the immunoglobulin molecule, and anti-isotype antibodies are directed against the constant regions (Fig. 1). Anti-anti-idiotype antibodies can also be produced. These recognize the binding region of the anti-idiotype antibody; thus, the antigen-binding region of an anti-anti-idiotype antibody resembles the antigen that elicited the original anti-idiotype HAMA *(78, 80)*. Additionally, the possibility exists for the formation of antibodies with specificity for antigens or neoantigens on the conjugated monoclonal antibody, e.g., anti-ricin antibodies *(54)*, or with specificity for a chimeric antibody *(81, 82)*.

Generally, isotype antibodies may be more common than idiotype antibodies. For example, in one study involving 141 patients, 29% were positive for HAMA after treatment with $^{99\,m}$Tc-BW 431/26. In 80% of these positive patients, the HAMA response was predominantly anti-isotypic, and in 20% it was predominantly anti-idiotypic *(57)*. In contrast, a study of a group of nine patients who developed HAMA 7–15 days after beginning treatment with B-E8, an $IgG_1$ directed against interleukin-1 revealed that all nine of the patients developed IgG anti-idiotype antibodies against B-E8. Four of the patients also developed IgM anti-idiotype antibodies *(50)*.

### MAGNITUDE AND DURATION OF RESPONSE

The magnitude and duration of an HAMA response shows great variability, and serum concentrations in the microgram per liter to gram per liter range have been detected *(83, 84)*. Anti-animal antibodies can persist in blood for several months after exposure to mouse immunoglobulin. For example, in one study *(85)*, an IgG HAMA was still detectable after 10 months, and in another study *(86)*, it was detectable up to ~30 months after immunoscintigraphy. In patients who have devel-

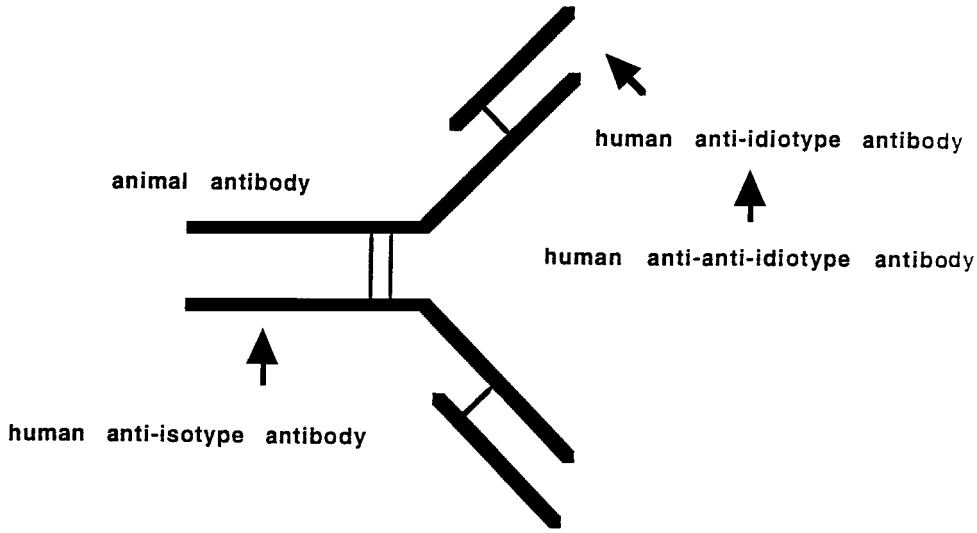


Fig. 1. Structure of IgG molecule and specificity of anti-animal antibodies.

oped an human anti-animal antibody response, B memory cells that express the antibody would presumably remain for years and would be activated upon reexposure to the antigenic stimulus.

### PREVALENCE OF HAMA AND ANTI-ANIMAL ANTIBODIES

The true number of people positive for anti-mouse antibodies is not known, and estimates vary widely (<1–80%) *(87–93)*. One problem has been the choice of method to detect anti-animal antibodies. There is no universal assay for this type of antibody because the antigen causing the human anti-animal antibody response in any given patient is usually unknown. For example, although HAMA assays may be able to detect anti-isotype antibodies, anti-idiotypic antibodies may escape detection. Table 3 *(87–93)* summarizes the results of a series of studies designed to detect HAMA and other anti-animal antibodies.

### CLINICAL SIGNIFICANCE AND CONSEQUENCES OF HAMA

It is not surprising that the administration of a foreign protein may be accompanied by some adverse reactions. Although uncommon, the spectrum of reported adverse reactions to intravenous, intraperitoneal, or subcutaneous mouse-derived agents includes allergic reactions (incidence, 0.3 in 1000) *(78, 94)*, anaphylactic shock *(95)*, generalized pain, hyponatremia, fever, rigors, chills, rash, paresthesias, weakness, chronic refractory postural hypotension *(70)*, and serum sickness *(96, 97)*. No relationship has been found between the adverse reactions and the development of a HAMA (IgG or IgM) *(78)*.

Preexisting HAMAs can also interfere with mouse monoclonal antibody therapy or imaging by inactivation or by complexation with the administered antibody. Rapid clearance of the complexed agent neutralizes its therapeutic effectiveness *(53, 79, 98)*. Sensitization to OKT3 has been studied and a relationship established between sensitization and mortality and/or allograft loss. The concentrations of OKT3 were followed, and failure to achieve steady-state or declining concentrations was equated with sensitization, subsequently demonstrated by the detection of HAMAs in six of the seven patients tested *(53)*. A further consequence of anti-animal antibodies is unnecessary medical intervention or medical or surgical procedures because of false-positive test results,

**Table 3. Studies to assess prevalence of anti-animal antibodies.**

| Prevalence | Population | Ref. |
|---|---|---|
| Human anti-mouse IgG | | |
| 76% | 67 blood donors | *87* |
| 80% | 10 infants | *87* |
| 0.72% | 10 000 blood donors | *88* |
| 9.12% | 1008 blood donors | *89* |
| Human anti-rabbit IgG | | |
| 5% | | *90* |
| 0.13% | 10 261 neonates | *38* |
| 0.09% | 75 734 neonates | *89* |
| 0.3% | 9241 infants | *37* |
| Human anti-chicken IgG | | |
| 0% | 150 patients and blood donors | *91* |
| Human anti-bovine albumin | | |
| 43% | 28 healthy subjects | *92* |
| Human anti-bovine IgG | | |
| 7% | Blood donors | *93* |
| Human anti-sheep IgG | | |
| 7% | Blood donors | *93* |
| Human anti-guinea pig IgG | | |
| 0.51% | Blood donors | *4* |
| Multivalent antibody-binding substances[a] | | |
| 40% | 668 blood donors, laboratory personnel, and patients | *5* |

[a] Antibody-binding substances are substances that react with a capture and a labeled detection antibody to form a sandwich.

particularly from tests for cancer markers (see the section on immunosuppressant therapy) *(8)*.

One potential benefit of a HAMA response is the induction of anti-anti-idiotype antibodies *(86, 99, 100)*. These should be reactive with the original target antigen of the infused antibody and thus would be reactive with antigen-expressing cells (e.g., tumor cells) and provide a therapeutic effect.

### ASSAYS FOR HAMA

The measurement of HAMA is important for the identification of specimens that may give falsely increased results in two-site assays and is also therapeutically important for assessing possible complications of repeated administration of mouse monoclonal antibodies. In it's "points to consider" document on monoclonal antibody products for human use, the FDA makes specific recommendations on monitoring of the development of HAMAs *(101)*, i.e., "develop assays to detect human immunoglobulins against humanized or primatized antibodies, immunonuclides, immunotoxins, their individual components, and neoantigens formed by the linked antibody/toxin/nuclide".

HAMA assay designs vary widely and include direct assays for immune complexes, immunofluorescence tests, immunofluorescence inhibition tests, IRMAs, ELISAs, reverse ELISA assays *(53, 83, 102, 103)*, and dot blotting *(104)*. Direct assays use HPLC to measure the immune complexes formed when serum is incubated with radiolabeled antigen (monoclonal antibody) infused into the patient. Other assays use either the same monoclonal antibody or a polyclonal antibody for capture and detection. Alternatively, a mouse antibody (monoclonal or polyclonal) is used for capture HAMAs, and an anti-species antibody is the detection antibody *(105)*.

The type of HAMA detected will depend on the assay design. For example, if the capture, detection, and infused antibodies are identical, then the assay will detect isotypic and idiotypic HAMAs. If an irrelevant monoclonal antibody of the same isotype as the infused antibody is used as the capture antibody, then the assay will detect predominantly isotypic HAMAs. The variability in HAMA results between different types of assays has been assessed by the distribution of panels of specimens to laboratories in the US and Europe, and these surveys revealed significant intermethod and interlaboratory differences in HAMA results *(106–108)*. The calibrators for HAMA assays also vary, and include baboon anti-mouse IgG, serum, or plasma from patients infused with monoclonal antibodies. Lack of standardization is a key factor in the poor intermethod and interlaboratory comparability of HAMA data.

Currently, there are six HAMA assays available in kit form: ImmuSTRIP HAMA (Immunomedics), ETI-HAMAK (Sorin Biomedica) *(109, 110)*, HAMA-ELISA medac (Medac), HAMA RIA (Scantibodies Laboratory), Ideal HAMA ELISA (AIPCO), and Enzygnost HAMA (Behringwerke). All except one of the assays are ELISAs. The Immunomedics assay uses mouse IgG immobilized to a plastic surface as the capture antigen and a mouse IgG-horseradish peroxidase conjugate to detect captured HAMA. The Sorin Biomedica test uses an immobilized mouse monoclonal antibody ($IgG_1$) and a goat anti-human IgG-horseradish peroxidase conjugate. In the Behring test, either IgG or IgM HAMA can be detected. The mouse monoclonal antibody ($IgG_1$) supplied with the kit or the mouse monoclonal antibody administered to the patient is immobilized on the inside surface of a plastic microwell (capture antigen), and the HAMA is detected with a goat anti-human IgG- or IgM-horseradish peroxidase conjugate. A simple point-of-care type test would be useful for the rapid assessment of specimens suspected of containing HAMAs. At one time, Sangstat produced such a device, but it has since been withdrawn from the market *(111)*. An alternative strategy now is to utilize a pregnancy test kit. A qualitative HAMA result can be obtained using the Tandem® ICON® ImmunoConcentration® human chorionic gonadotropin (hCG) assay. This has a negative control zone that is coated with mouse IgG specifically to detect anti-animal antibodies that might invalidate the hCG test; if this zone develops a color (positive response), this indicates that the sample is positive for HAMA. However, this assay may be relatively insensitive to HAMA because blocking agents (mouse, rat, or bovine) are included in the sample diluent. It should also be remembered that the device was not specifically designed for this purpose.

### MECHANISM OF INTERFERENCE IN IMMUNOASSAYS

In two-site (sandwich) immunoassays, HAMAs present in a serum sample can interfere in clinical assays by bridging between the mouse immunoglobulin capture antibody and the mouse immunoglobulin conjugate (Fig. 2); this produces a false-positive result (Table 4) *(112–150)*. False-negative results attributable to HAMA are also encountered in two-site assays, and this presumably is the result of the HAMA reacting with one of the assay reagents (immobilized antibody or the conjugate) and preventing reaction with the analyte (Fig. 2). A correlation ($r = 0.885$) has been shown between HAMAs and false positivity in a CA 125 assay *(122)*. However, there were some outliers in which an increased HAMA concentration was not associated with a increased concentrations of CA 125, indicating a more complex mechanism for the interference (e.g., HAMA may have greater reactivity with the monoclonal antibody in the HAMA assay than with monoclonal antibody in the CA 125 assay).

Interference has also been reported in solid-phase competitive binding assays as a result of blocking of the capture antibody binding site *(93)*. The high affinity of the antigen and labeled antigen for the capture antibody, compared with the human anti-animal antibody, minimizes interference in competitive binding assays. However, interferences can occur if the anti-animal antibody is

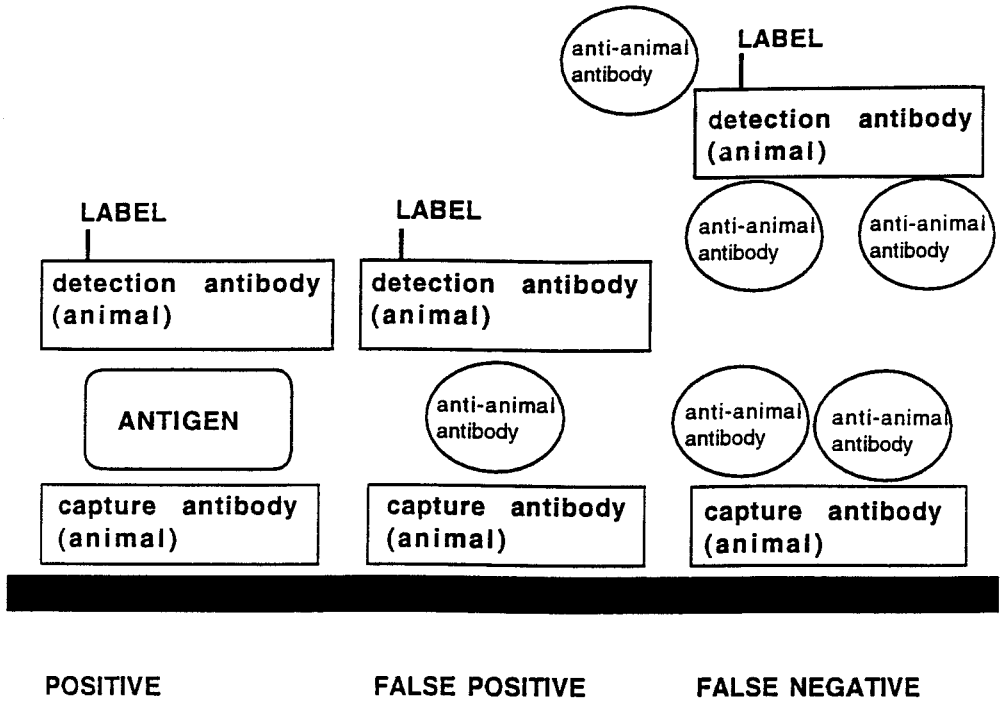


Fig. 2. Mechanism of HAMA interference in two-site assays.

present in a high concentration or if a large amount of sample is used in the assay. An interference has also been reported in a competitive binding assay that used a double antibody technique for separation of the bound from the free labeled fractions *(93)*.

### Antibodies to Other Species

Circulating antibodies with specificities for a wide range of animal immunoglobulins have been documented, but little is known of their prevalence. Antibodies against rabbit and goat immunoglobulins are particularly important because these animals are used as the sources of antisera for immunoassay reagents. Antibodies with other specificities can also be problematic in immunoassays because of cross-reactivity *(151)*.

**Table 4. Assay interferences and interference studies.**

| Assay | Ref. |
|---|---|
| CA 125 | *86, 112–120* |
| CEA | *121–125* |
| CK-MB | *126, 127* |
| Erythropoietin | *128* |
| Estradiol | *129* |
| Free thyroxine | *130* |
| FSH | *131, 132* |
| Hepatitis B surface antigen | *4, 125* |
| hCG | *76, 125, 133–137* |
| Luteinizing hormone | *125, 131* |
| Progesterone | *129* |
| Prolactin | *136, 138* |
| Rubella-specific IgM | *139, 140* |
| Thyroxine | *130* |
| Triiodothyronine | *130* |
| Troponin I | *141* |
| TSH | *38, 90, 125, 142–150* |

#### HUMAN ANTI-RABBIT ANTIBODY

Rabbit anti-thymocyte globulin is an immunosuppressant, and anti-rabbit antibodies develop in patients who receive this therapy. In one study of a group of 32 renal transplant patients, all developed human anti-rabbit antibodies (HARAs; 144.6 ± 33.7 mg/L IgA, 187.5 ± 100 mg/L IgG, and 44.9 ± 12.6 mg/L IgM), and in the case of the IgG anti-rabbit antibody, these persisted for 2–12 months *(152)*. The adverse consequences of an unrecognized human anti-animal antibody interference is dramatically illustrated by two case reports of a HARA interference *(39)*. In the first case, increased concentrations of serum hCG in a 41-year-old woman led to an unnecessary laparoscopic examination. Her serum hCG had been measured with an RIA based on antibodies raised in rabbits, and reanalysis of the patient's specimens with a goat antibody-based assay revealed normal concentrations of hCG in all samples. A more disturbing case was that of a woman who presented because of infertility and amenorrhea. High serum follicle-stimulating hormone (FSH) values were noted, and this led to a series of unnecessary diagnostic procedures, including laparoscopy, laparotomy, and an ovarian biopsy. Reanalysis of her samples with a goat antibody-based assay gave normal values for FSH. Both patients kept rabbits as pets, and it was surmised that this was the source of antigen.

Other cases of HARA interferences (blockable with rabbit serum or IgG) include falsely increased thyrotropin (TSH) attributable to placental transfer from HARA-positive mothers *(37, 153)*, increased luteinizing hormone and FSH attributable to vaccination *(32)*, and unusual treatments [e.g., subcutaneous injections with antireticulocytoxique, a serum obtained by injecting rabbits with homogenates of human bone marrow and spleen *(34)*].

### human anti-goat antibody

An interference attributable to serum human anti-goat antibodies was uncovered in serum from an 84-year-old woman following discordant creatine kinase (CK) isoenzyme results obtained with an immunoassay (Stratus CK-MB assay result, 12–15 $\mu$g/L) and by electrophoresis (>95% MM isoenzyme, no detectable MB). The addition of mouse IgG was without effect on the assay results (indicating that the sample probably did not contain HAMA), but the addition of normal goat serum reduced the measured CK-MB to <1.7 $\mu$g/L, suggesting that a human anti-goat antibody was the most likely cause of the interference. The Stratus CK-MB assay includes goat IgG as a component of the anti-CK-MB-alkaline phosphatase conjugate reagent. Presumably the human anti-goat antibody reacted with the goat IgG, and the resulting immune complexes trapped conjugate on the Stratus assay tab to give a false-positive result. This was supported by the finding that removal of the goat IgG from the conjugate eliminated the interference *(154)*. Anti-animal antibodies reactive with goat as well as mouse IgG have also been described *(118)*.

### human anti-sheep antibody

Digibind™ is a sheep anti-digoxin-Fab widely used to treat digoxin poisoning, but there are no reports of the formation of anti-sheep antibodies following this type of treatment. This may be because it is a Fab fragment and is rapidly removed from the circulation. An interference in a RIA for $\alpha$-fetoprotein because of human anti-sheep antibody has been described *(93)*. A 7% prevalence of human anti-sheep antibody in a blood donor population was found, and it was suggested that this was not attributable to occupational exposure (shepherds, slaughterhouse workers, or butchers), but to immunization via the gut with bovine immunoglobulin, which is cross-reactive with sheep immunoglobulin.

### human anti-cow antibody

Human anti-cow antibody interferences have been reported in the serum of three patients tested for thyroxine, free thyroxine, and TSH with the enhanced chemiluminescent Amerlite assays. Addition of bovine $\gamma$-globulin (final concentration, 10 g/L) eliminated the interferences *(155)*.

### human anti-pig antibody

Human anti-porcine antibodies have been detected in hemophiliacs receiving porcine factor VIII, but no assay interferences were noted *(156)*.

### human anti-rat antibody

This type of human anti-animal antibody was not detected during the treatment of 15 allogeneic bone marrow transplant patients with an anti-CD25 rat monoclonal antibody (33B3.1) for prevention of graft vs host disease *(74)*, and no analytical interferences attributable anti-rat antibodies have been reported.

### human anti-horse antibody

The formation of anti-horse antibodies as a result of treatment with an equine anti-thymocyte globulin immunosuppressant has been recognized for a long time. In one study, anti-equine antibodies developed in 4 of 27 cardiac transplant patients treated with equine anti-thymocyte globulin *(157)*.

### human anti-chimeric antibody

Human anti-chimeric antibodies have been detected in patients treated with chimeric antibodies *(81)*, although in other studies (e.g., studies of the treatment of multiple myeloma patients with chimeric human anti-interleukin-6 antibodies), this type of human anti-animal antibody was not detected *(82)*.

### antibodies with mixed specificity

There is considerable protein sequence homology between IgG molecules from different animal species. Thus, it is not surprising that anti-animal antibodies are cross-reactive with a range of animal immunoglobulins. Cross-reactivity of anti-animal antibodies has been illustrated by studies in two healthy males with spuriously increased serum luteinizing hormone concentrations *(151)*. This anti-animal interference was blocked with equivalent efficacy by mouse, sheep, or goat serum. It was also blocked with mouse $IgG_1$, mouse $IgG_{2a}$, and rat IgG. Another study investigated interferences in a two-site CK-MB assay and showed the broad reactivity of interfering globulins to nonimmune serum from a diverse range of animals (Table 5) *(89)*.

Anti-animal antibodies that cross-react with mouse IgG, causing a false-positive result in an $\alpha$-fetoprotein assay, have been attributed to treatment with unconventional drug preparations, specifically Wobenzy (MUCOS Pharma), a formulation of partly plant and animal origins. Repeated administration of this preparation was thought to have immunized the patient to produce anti-animal antibodies that cross-reacted with mouse IgG *(158)*.

An intriguing type of anti-animal interference in an enzyme immunoassay for TSH (peroxidase label) has been described in a series of 14 specimens *(159)*. No interference was found in a RIA using an identical mouse monoclonal antibody capture antibody. The interference

**Table 5. Effect of nonimmune sera on apparent CK-MB concentrations.[a]**

| Species | Serum samples blocked,[b] % |
|---|---|
| Mouse | 100 |
| Sheep | 78 |
| Cow | 78 |
| Guinea pig | 69 |
| Rat | 70 |
| Rabbit | 25 |
| Cat | 3 |
| Dog | 3 |
| Pigeon | 0 |

[a] Adapted from Thompson et al. *(89)*.
[b] CK-MB decreased >80%.

was inhibited by high concentrations of mouse IgG and blocked by anti-human IgM. The authors speculated that the anti-animal antibodies recognized epitopes on the peroxidase label or epitopes on the antibody exposed or modified by enzyme labeling.

## Strategies to Eliminate Anti-Animal Antibodies and Antibody Interferences

Several strategies have been developed to prevent the development of anti-animal antibodies in patients receiving animal-derived agents. These range from structural modification of the agent to suppression of the patient's immune system. Procedures are also available to remove or block anti-animal antibodies that may be present in specimens before analysis. In addition, non-cross-reacting chicken antibodies have been advocated as alternatives to antibodies from other species as the source of immunoassay reagents.

### PREVENTION

Immunosuppressant therapy. One method of minimizing the development of HAMA has been to treat patients with immunosuppressive drugs such as cyclosporin A, cyclophosphamide, azithioprine, or deoxyspergualin before, during, and after the administration of mouse antibody agents *(79, 84, 160, 161)*. In a recent study, a series of 13 patients were given cyclosporin A starting 2 days before treatment with a $^{99\,m}$Tc-labeled F(ab′)$_2$ or Fab *(161)*. Six to 9 days later, $^{186}$Re-F(ab′)$_2$ or intact antibody was administered, and cyclosporin A treatment was continued for an additional 14 days. In 5 of 13 patients (mean cyclosporin A, 726 μg/L), no HAMA was detected for up to 8 weeks, whereas the remaining 8 patients with lower cyclosporin A concentrations (mean, 364 μg/L) became HAMA positive. In control experiments involving patients who were not given cyclosporin A, 86–100% of patients developed a HAMA response *(161)*. Similarly, in a series of patients treated with two courses of radiolabeled anti-carcinoembryonic antigen (CEA), the mean serum HAMA was 3.5 mg/L after 2 weeks in patients treated with cyclosporin A vs 1998 mg/L in patients not given this immunosuppressant *(84)*. Deoxyspergualin is also effective in suppressing the HAMA response. In patients with advanced cancers treated with the antibody L6, two-thirds developed HAMAs in the original trial, but when L6 was administered in combination with deoxyspergualin, only 2 of 24 patients developed HAMAs (both had low serum HAMA concentrations, 160 and 181 μg/L) *(160)*.

Antibody fragments. The immunogenicity of an immunoglobulin molecule can be reduced by removing the Fc portion. The resulting Fab or F(ab′)$_2$ fragments have been shown to be less immunogenic than the intact IgG molecule *(109, 162, 163)*, although the incidence of HAMA positivity increases with multiple therapies for intact or fragments of mouse monoclonal IgG *(109)*.

Humanized and chimeric antibodies. One way of overcoming the antigenicity of mouse monoclonal antibodies has been to "humanize" the immunoglobulin molecule. This can be achieved using genetic engineering techniques to combine mouse complementary determining regions and human framework and constant regions or human constant regions with mouse framework and complementary determining regions *(164–169)*. A difficulty encountered with the humanization strategy is that an IgG molecule is still potentially antigenic; hence, an immune response will produce human anti-human antibodies. In one study, 2 of 53 patients given 88BV59, an IgGk directed against the tumor-associated antigen CTA16.88 (homologous to cytokeratins 8, 18, and 19), developed a low titer of human anti-human antibodies 1–3 months after a single infusion of the antibody *(116)*. An analytical interference by human anti-human antibodies may be possible as a result of cross-reactivity, but is as yet unreported.

Polyethyleneglycolylation. Coating the surface of a macromolecule with water-soluble polyethylene glycol (PEG) or monomethoxy PEG (mPEG) molecules can lead to beneficial alterations in their properties, e.g., reduced clearance and reduced immunogenicity, enhanced tissue localization, specificity, potency, and stability *(170–173)*. For example, immunogenicity reduction through mPEGylation has been shown in animal studies with the murine antibody W3/25 *(173)*. This chemical "stealth" type technology offers a potential route to reducing or eliminating HAMAs in patients receiving mouse monoclonal antibodies.

### BLOCKING AND REMOVAL

Considerations for methods designed to block or to remove a anti-animal interference are ease of use, effectiveness, applicability, cost, and convenience. Many of the available methods have deficiencies in one of these areas.

The blocking agent can be included in the assay (e.g., in the assay diluent), or the sample can be pretreated before assay. Nonimmune serum *(4, 126, 174, 175)*, polyclonal IgG *(121, 124, 126)*, polymerized IgG *(88)*, nonimmune (irrelevant) mouse monoclonals *(103)*, and a mixture of monoclonal antibodies *(124)* or fragments of IgG [Fc, Fab, F(ab′)$_2$] *(142)* from the same species used to raise the

reagent antibodies are commonly used as blocking agents *(103, 121, 174)*. The effectiveness of added blocking agent depends on the concentration and class or subclass, specificity, and valence of the human anti-animal antibody and the species and subclass of the blocker *(142)*. There are examples of HAMA interferences that were either not blocked or only partially blocked by mouse IgG. In one case, blocking could only be achieved by low temperature incubation with a high concentration of the monoclonal antibody administered to the patient *(125)*.

Several blocking reagents are available commercially: Immunoglobulin Inhibiting Reagent (IIR; Bioreclamation) *(176)*, Heterophilic Blocking Reagent (HBR; Scantibodies), Heteroblock (mixture of active and passive blocking reagents; Omega Biologicals), and MAB 33 (monoclonal $IgG_1$) and Poly MAB 33 (polymeric monoclonal $IgG_1$/Fab; Boehringer Mannheim). IIR is a proprietary formulation of immunoglobulins with a high affinity for anti-animal antibodies ($10^9$ L/mol), and HBR is monoclonal mouse anti-human IgM. In conventional blocking procedures, the blocking depends on the binding constant of the human anti-animal antibody with the added reagent (typically $10^6$ L/mol). In contrast, reagents such as IIR and HBR are directed specifically against any IgM, not only those with anti-animal specificity, and have a higher binding affinity ($10^9$ L/mol) for an human anti-animal antibody than does an anti-animal for a nonspecific blocking agent. Consequently, these reagents can be used at lower concentrations and have superior blocking kinetics compared with nonspecific blocking reagents. In a comparative study of IIR vs a polymerized nonimmune (irrelevant) monoclonal (MAK-33) and nonspecific mouse IgG in a CA 125 assay, only IIR eliminated all interferences *(103)*. In a study of HBR, it was shown to be effective in blocking anti-animal interference in a serum CK-MB assay. HBR also caused small changes (range, −6.8% to 11.5%) in the concentration of CK-MB in control specimens, and this was attributed to the intrinsic anti-immunoglobulin reactivity of the reagent *(177)*. One alternative to the HBR product are the Heterophilic Blocking Tubes (Scantibodies), which contain proprietary predispensed and lyophilized specific binders to inactivate anti-animal antibodies *(178)*.

Immunoextraction using murine monoclonal antibody adsorbed onto vinylidene fluoride floccules *(179)* or protein G immobilized on Sepharose beads *(103)* has also been effectively used to remove HAMA interferences from samples in a CEA and a CA 125 assay, respectively. Alternatively, anti-animal interferents can be removed by precipitation with PEG 6000 *(136, 180, 181)*. Chromatography is also effective in removing interferents. For example, protein A, protein G, cation-exchange, or gel filtration chromatography was used in a CA 125 assay for samples that could not be blocked with mouse serum or purified mouse antibody *(117)*.

A combination of heat and acid treatment of samples is of limited utility because few analytes are sufficiently stable to survive these antibody-denaturing conditions. This procedure is used mainly as a sample pretreatment procedure for CEA assays (70 °C or 90 °C and acetate buffer, pH 5) *(122, 181)*. Optimization of these conditions is required for individual cancer markers, and for a CA 72-4 assay, the combination of 90 °C and Bis-Tris, pH 6.5, was most effective *(182, 183)*.

### ASSAY REDESIGN

One solution to the problem of human anti-animal antibody interferences in two-site assays is to use Fab or the $F(ab')_2$ fragment instead of intact immunoglobulin as the capture and detection antibodies. This eliminates interference from anti-animal antibodies with specificity for the Fc portion of an IgG antibody reagent *(126, 142)*. Another strategy is to use chimeric monoclonal antibodies as assay reagents. These are now used in some Boehringer Mannheim immunoassays (e.g., ES and Elecsys TSH assays and the Elecsys CEA assay), either as the capture antibody or the labeled antibody *(184)*. These chimeric antibodies are human antibodies in which the variable regions are replaced with the corresponding parts of a non-human antibody (e.g., mouse or rat) of the desired specificity. In this way, interferences by anti-mouse and other anti-animal antibodies are eliminated.

Another alternative is to use antibodies raised in chickens for one or both of the antibody reagents *(91)*. Mammalian and chicken IgG have no cross-reactivity; thus, chicken antibodies are unlikely to react with anti-animal antibodies. Chicken antibody-based assays have been tested using a rabbit anti-mouse antibody (HAMA surrogate) and with sera from HAMA-positive patients (treated with monoclonal antibody 17-1A). No false positives were observed when at least one of the antibodies (capture or detection) was a chicken antibody. However, chicken antibodies have low affinities, and there are currently no monoclonals, thus preventing two-site monoclonal assay strategies.

## Conclusion

Anti-animal antibodies often go unnoticed, to the detriment of patient care. Fortunately, there is a growing awareness on the part of laboratory staff and clinicians of the problems caused by this type of interference *(46, 185–186)*. Each member of the healthcare team has a role to play in guarding against the adverse analytical effects of anti-animal antibodies (Table 6). Clinicians should ensure that patients known to have such antibodies or at risk for developing such antibodies because of administration of animal-derived agents are clearly identified to the laboratory. They should also be aware that if the results of two-site immunoassay tests, particularly cancer marker and hormone tests, do not fit with the clinical picture, then this may be an indication of a human anti-animal antibody interference. Manufacturers of two-site immunoassay kits should take steps to minimize assay interferences, and some have already responded by reformulat-

**Table 6. Roles and responsibilities in guarding against the adverse effects of analytical interferences caused by circulating anti-animal antibodies.**

| | |
|---|---|
| Patient | |
| | Inform clinician of any prior exposure to animal-based therapeutic or diagnostic agents (e.g., imaging agents). |
| Clinician | |
| | Ask patient about prior exposure to animal-based therapeutic or diagnostic agents or exposure to animals (pets, animal husbandry). As appropriate, provide this information to the laboratory. |
| Laboratory | |
| | Flag immunoassay results from patients known to have been exposed to animal-based agents. Provide follow-up to clinical staff on significance of the possible interferences and strategies to identify, confirm, and overcome the interferences. |
| | Investigate and confirm anti-animal interferences by adding blocking agents to samples and retesting, performing serial dilution studies, testing for presence of anti-animal antibodies, and retesting with another analytical method (antibodies from another animal species). |

ing assay reagents and including warnings about HAMA interferences in package inserts. Laboratories should develop plans for investigating and confirming anti-animal interferences. Dilution experiments will often flag a possible interference because samples containing anti-animal antibodies do not give proportional results. Reanalysis of samples after incubation with animal protein (e.g., mouse IgG) or animal serum can also help to confirm an interference. Another strategy to guard against false-positive and false-negative results attributable to anti-animal antibodies is to test all samples for the presence these antibodies. This would be an expensive undertaking, and currently this type of analysis is reserved for identifying the presence of an human anti-animal antibody.

This review was prepared as part of the activity of the IFCC Committee on Advanced Technology (Prof. P. Bonini, Dr. G. Hoffmann, Prof. K. Yasuda, Prof. W. Godolphin, and Prof. M. Sasaki). I thank Drs. Stanley Levinson, Paul M Kaladas, and Helmut Lenz for critical review.

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