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Interferences in Immunoassays

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1. Introduction

Interference in immunoassays is a serious but underestimated problem (Ismail et al, 2002a). Interference is defined as "the effect of a substance present in the sample that alters the correct value of the result, usually expressed as concentration or activity, for an analyte" (Kroll & Elin, 1994). Immunoassays are analytically sensitive and measurements can frequently performed without prior extraction. However, immunoassays may lack adequate specificity and accuracy. Specificity of an immunoassay does not only depend on the binding property of the antibody but also the composition of the antigen and its matrix is important. Specificity can also be influenced by reagent composition and immunoassay format. Substances that alter the measurable concentration of the analyte in the sample or alter antibody binding can potentially result in assay interference (Tate & Ward, 2004).

Interference can be analyte-dependent or analyte–independent and it may increase (positive interference) or decrease (negative interference) the measured result. The common interferences of hemolysis, icterus, lipemia, effects of anticoagulants and sample storage are independent of the analyte concentration. Analyte-dependent interferences in immunoassays are caused by interaction between components in the sample with one or more reagent antibodies. They include heterophilic antibodies, human anti-animal antibodies, auto-analyte antibodies, rheumatoid factor and other proteins. Interferences may lead to falsely elevated or falsely depressed analyte concentration depending on the nature of the interfering antibody or the assay design (reagent limited versus reagent excess assays). The magnitude of the effect depends on the concentration of the interferant, but it is not necessarily directly proportional. It can also lead to discordant results between assay systems (Selby, 1999; Tate & Ward, 2004).

Interference can have important clinical consequences and may lead to unnecessary clinical investigation as well as inappropriate treatment with potentially unfavorable outcome for the patient (Ismail & Barth, 2001). It is important to recognize interference in immunoassays and put procedures in place to identify them wherever possible (Kricka, 2000).

2. Nature of Interference

Endogenous interfering substances can occur in both healthy and pathological patient samples. Sample properties are unique for each patient. Interference is caused by interaction with one or more steps in the immunoassay procedure and the analyte concentration or the antibody binding is influenced (Davies, 2005). Unsuspected binding protein(s) in the individual can interfere with the reaction between analyte and assay antibodies. In reagent-

excess assays, like the common two-site immunometric assay (IMA), there is an increased chance of a cross-reactant forming a bridge between the two antibodies. Conformational changes to antigens can be induced by antibodies which may alter the specificity of antibodies. For these reasons there may be a higher prevalence of unpredictable cross-reaction in IMAs than in the single-site antigen-antibody reaction in reagent-limited assays (Boscato & Stuart, 1986). Exogenous antibodies given to a patient for therapy may also compete with the assay antibody for the analyte and disturb the antigen-antibody reaction resulting in immunoassay interference, e.g., administration of Fab fragments derived from anti-digoxin antibodies (Digibind) (Hursting et al., 1997).

Exogenous interferences are any interference caused by the introduction of external factors or conditions, in vivo or in vitro, not normally present in native, properly collected and stored samples. For example, hemolysis, lipemia, icterus, blood collection tube additives,
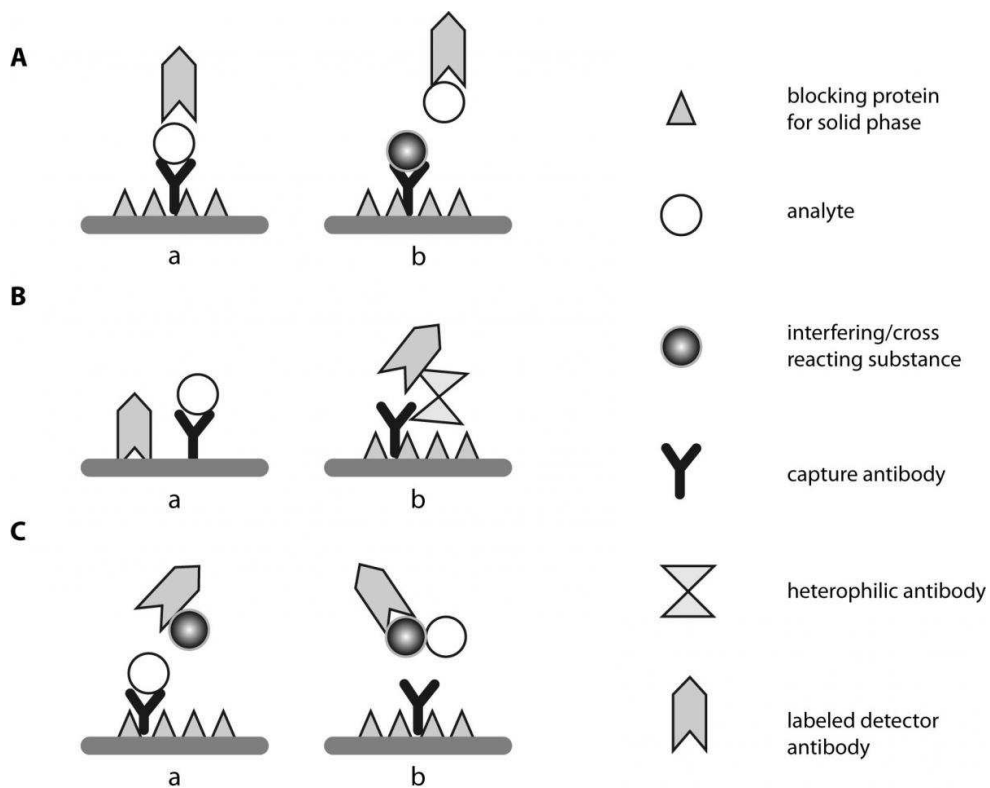

Fig. 1. Different interferences in immunometric immunoassays: Aa - assay without any interference; Ab - cross-reactivity of an interfering substance with capture antibody, resulting in false negative result; B - positive interference: Ba - unspecific binding of labelled detector antibody to a not blocked solid phase; Bb - "bridge" binding by heterophilic antibodies or HAMA, respectively; C - negative interference: Ca - change of sterical conformation after binding of interfering protein to Fc fragment of detector antibody Cb - masking of the epitope on analyte surface by a protein of the sample (Dodig, 2009).

administration of radioactive or fluorescent compounds, drugs, herbal medicines, nutritional supplements, sample storage and transport are all exogenous interferences that can adversely affect immunoassays (Selby, 1999).

Figure 1 summaries the possible interference mechanisms in IMAs.

3. Cross-reactivity

Cross-reactivity is the most common interference in immunoassays, but mostly in competitive assays. It is a non-specific influence of substances in a sample that structurally resemble the analyte (carry similar or the same epitopes as the analyte) and compete for binding site on antibody, resulting in over- or underestimation of analyte concentration. Cross-reaction is a problem in diagnostic immunoassays where endogenous molecules with a similar structure to the measured analyte exist or where metabolites of the analyte have common cross-reacting epitopes, and where there is administration of structurally similar medications (Kroll & Elin, 1994). The most common examples can be seen during determinations of hormone concentration, drugs and allergen-specific IgE. Hormones TSH (thyroid-stimulating hormone), LH (luteinising hormone) and hCG (human chorionic gonadotrophin) carry an analogue α-chain, and the β-chain determines the specificity of the respective hormone. Early hCG immunoassays cross-react with LH, but the development of more specific antibodies has led to most of today's assays for hCG having little or no cross-reaction with LH (Thomas & Segers, 1985). However, cross-reactivity with drugs and their metabolites is still a problem for the measurements of steroids which have an identical structure. For example, cortisol assays can show significant cross-reactivity with fludrocortisone derivates and result in falsely elevated cortisol levels in patients using these drugs (Berthod et al., 1988). The problem of cross-reactivity in active vitamin D (1,25(OH)$_2$D) determination due to possible positive interference of 25(OH) D is well known (Lai et al., 2010).

In competitive immunoassays for drugs of abuse screening, positive interference may result from medications or their metabolites that have similar chemical structures (Lewis et al., 1998).

In the regular monitoring of the transplant anti-rejection drug cyclosporine A in whole blood for dosage adjustment in patients after heart or liver transplantation, only the concentration of the parent drug should be used. Immunoassays for cyclosporine A show cross-reactivity for cyclosporine metabolites with levels up to 174% higher in individual patients compared with the HPLC reference method (Steimer, 1999).

In digoxin immunoassays, the presence of digoxin-like immunoreactive factors that are commonly found in renal failure, liver disease and hypertension, cause interference by cross-reaction (Dasqupta, 2006). Falsely suppression of results can also occur when a cross-reacting substance is present in the sample and during the wash or separation step the dissociation rate for the cross-reactant is greater than that for the analyte (Valdes & Jortani, 2002).

Interference due to cross-reactivity is highly dependent on assay specificity, which is not the focus of this chapter.

4. Alteration of the measurable concentration in the sample

4.1 Pre-analytical factors

All factors associated with the constituents of the sample are termed pre-analytical factors (Selby, 1999).

4.1.1 Blood collection

Blood collection tubes are not inert containers but have several constituents, including substances in and/or applied to rubber stoppers, tube wall material, surfactants, anticoagulants, separator gels and clot activators that can potentially interfere with immunoassays (Weber, 1990). Many laboratories have converted from glass to plastic collection tubes for convenience. Plastic blood collection tubes have been shown to be suitable for routine clinical chemistry analytes, hormone analysis and therapeutic drug monitoring (Wilde, 2005). But some low-molecular weight organic substances released by plastic tubes could interfere in some assays. The physical masking of the antibody by lipids and silicone oils present in some blood collection devices or tubes can physically interfere with Ag-Ab binding. The water-soluble silicone polymer coating the interior of serum separator tubes can interfere negatively with avidin-biotin binding in an IRMA for thyrotropin, prolactin, and hCG (Wickus et al, 1992). Conversely, silicone formed a complex with C-reactive protein (CRP) that enhanced the Ag-Ab reaction in the Vitros CRP assay resulting in falsely elevated results (Chang et al., 2003).

4.1.2 Sample type

For many immunoassays, serum is the matrix of choice; however, plasma can be a very useful alternative, as it eliminates the extra time needed for clotting, thereby reducing the overall pre-analytical time (Selby, 1999). Tubes containing anticoagulants must be filled to the mark, otherwise the concentration of the anticoagulants will be too high and this may affect the assay system, particularly the Ag-Ab characteristics. If several specimens are to be drawn at the same time the plain tube should always be first filled. The recommended order to fill being plain tube, citrate, lithium heparin, EDTA and finally fluoride/potassium oxalate. Care must still be taken to avoid cross-contamination between different additive tubes (Wilde, 2005). Sample type can affect analyte concentration with different results for samples collected in lithium heparin, EDTA, and sodium fluoride/potassium oxalate or tubes without anticoagulant reported for some analytes, e.g., cardiac troponin, hormones (Evans et al., 2001). If plasma is used for immunoassay, care must be taken to select the appropriate anticoagulant. Anticoagulants added to specimens in appropriate concentrations to preserve certain analytes, may cause problems with the assay of other analytes. Heparin may interfere with some antibody-antigen reactions.

4.1.3 Hemolysis, lipemia and icterus

Immunoassays are mostly unaffected by hemolysis and icterus unlike other analytes measured by spectral or chemical means (Tate & Ward, 2004). However, hemolysis may be unacceptable for immunoassays of relatively labile analytes like insulin, glucagon, calcitonin, parathyroid hormone, ACTH and gastrin, due to the release of proteolytic enzymes from erythrocytes that degrade these analytes. Samples with any sign of hemolysis

are not acceptable for such assays. Because hemolysis may also interfere with some signal generation steps of different types of immunoassays, grossly hemolysed specimens should not be used. Lipemia can interfere in some immunoassays especially those using nephelometry and turbidimetry. Lipemia of serum or high levels of triglycerides, cholesterol or both may produce erroneous results in some assays by interfering with antigen binding, even when antibodies are linked to a solid support. Interferences by non-esterified fatty acids have been well documented for free thyroxine assays. Non-esterified fatty acids compete with thyroxine and its derivatives used as labels for endogenous protein binding sites and, depending on the assay format, may cause either falsely high or falsely low free thyroxine values. Binding of steroids may also be inhibited by non-esterified fatty acids. Hypertriglyceridaemia has been shown to cause falsely elevated results in some endocrine assays, using second antibody and polyethylene glycol separation techniques. Ideally, specimens should be collected from individuals following an overnight fast to reduce the immunoassay interference from lipids. Alternatively, ultracentrifugation but not dilution could be used to remove any excess lipids, or enzymatic cleavage by lipase may be used to treat samples before analysis. Excess bilirubin can also affect many different types of assays, including immunoassays (Wilde, 2005).

4.1.4 Stability and storage

Inappropriate specimen processing or storage can change the properties of a sample over time and affect immunoassay results. Most analytes are more stable when the sample is maintained in a cool or frozen condition. For some, especially the small peptide hormones, storage at -20°C and transportation in frozen state is necessary for reliable results. Such hormones include insulin, c peptide, gastrin, glucagon, ACTH and vitamin D (Wilde, 2005). For example, ACTH is reported to be stable in EDTA plasma at 4°C for only 18 hours compared with many other hormones that are stable for >120 hours (Ellis et al., 2003). Repeated freeze/thaw cycles can lead to denaturation, aggregation and loss of antigenicity of some proteins. Because EDTA chelates calcium and magnesium ions, which function as coenzymes for some proteases, blood specimens collected in EDTA are often more stable than serum or heparinised plasma. But elevated EDTA levels in the sample-reagent mixture, due to insufficient sample volume, can affect the activity of the alkaline phosphatase enzyme label used in chemiluminescence assays. Filling EDTA tubes to <50% affects intact parathyroid hormone and ACTH measurements by the Immulite assays (Glendenning et al., 2002). Some of the low mass polypeptide hormones such as ACTH, glucagon, gastrin and the gastrointestinal hormones are rapidly destroyed by enzymes present in blood and may require protection by addition of protease inhibitors (e.g., aprotonin) to the tube into which the blood sample is taken (Wilde, 2005).

4.1.5 Carryover

Integrated systems that combine clinical chemistry and immunoassay analysers are more and more used routinely. Sample to sample carryover is an inherent risk and can cause erroneously high test results for immunoassays (Armbruster & Alexander, 2006). Potential sample carryover due to inadequate washing or failure to detect a sample clot can also results in over- or under-estimation of values. If a sample to be assayed is preceded by a sample with a very high concentration of an analyte e.g. hCG, tumour markers, some of the

analyte from the first sample on the instrument probe may significantly increase the concentration of the analyte in the second sample.

4.2 Hormone binding proteins

Hormone binding globulins can alter the measurable analyte concentration in the sample either by removal or blocking of the analyte (Tate & Ward, 2004). Important endogenous binding globulins are albumin (because of its large concentration), sex hormone binding globulin (SHBG), thyroid binding globulin (TBG) and cortisol binding globulin (CBG). For total hormone measurement, it is essential to displace all bound hormone from endogenous binding sites and to prevent the binding of labelled hormone to the endogenous binding site. This can be done by solvent extraction, denaturation of the binding proteins, by adding blocking agents or by immunoaffinity extraction. For example, increased or decreased SHBG concentrations can interfere in direct assays for steroids testosterone (Slaats et al., 1987) and estradiol (Masters & Hähnel, 1989) and binding of cortisol to CBG can be minimized by denaturation of the binding protein or by addition of blocking agent. In free hormone measurement, displacement of analyte from endogenous hormone binding proteins, e.g., free thyroxin (FT4) displaced from thyroid binding globulin (TBG) by non-esterified free fatty acids (NEFA), can alter assay equilibrium and either increase or decrease the free analyte concentration (Nelson & Wilcox, 1996). These NEFAs can be generated in-vitro in non-frozen samples from patients receiving heparin, secondary to the induction of heparin-induced lipase activity. Increased serum triglyceride levels can accentuate this problem (Mendel et al., 1987).

4.3 Autoanalyte antibodies

Autoantibodies have been described that can cause interference for a number of analytes including thyroid hormones in both free and total forms (Symons, 1989), thyroglobulin (Spencer et al., 1998), insulin (Sapin, 1997), prolactin (Fahie-Wilson & Soule, 1997) and testosterone (Kuwahara, 1998). Positive or negative influence may occur, depending on whether the autoantibody-analyte complex partitions into the free or the bound analyte fraction. Interference from autoantibodies can occur in both immunoassay formats (Tate & Ward, 2004).

Autoantibodies against thyroid hormones, especially anti-T4 and anti-T3 antibodies, have been reported in patients with Hashimoto's thyroiditis, Graves' disease, hyperthyroidism after treatment, carcinoma, goitre and non-thyroid autoimmune conditions. These endogenous factors particularly interfere in total T4, free T4, total T3 and free T3 methods. Thyroid hormone antibody interferences are difficult to predict and can occur even with frequently used and well-characterised methods. Antibody prevalence depends on the detection method used: it is low in healthy subjects but may be as high as 10% in patients with autoimmune disease although only a minority of such samples demonstrate substantial thyroid assay interference (Després & Grant, 1998). Their presence should be suspected when FT4 and TSH results appear to be discordant to the clinical findings.

Interference is also a serious problem in Tg assays largely due to endogenous Tg antibodies (TgAb). Serum TgAbs are present in up to 25% of differentiated thyroid cancer (DTC) patients and in 10% of the general population. It is important to use a Tg method that

provides measurements that are concordant with the tumour status in DTC patients. IMA methods are prone to underestimate serum Tg when TgAb is present, increasing the risk that persistent or metastatic DTC will be missed. Because falsely low Tg results can occur by IMA and falsely elevated results by RIA, anti-Tg antibodies should be measured in all samples analysed for Tg and a possible interference should be retained in all TgAb positive samples (Spencer et al., 1998).

Anti-prolactin autoantibodies can be present in serum in the form of macroprolactin (macro-PRL). The presence of macro-PRL can cause macroprolactinemia with normal prolactin (PRL) concentrations and may lead to unnecessary medical or surgical procedures (De Schepper et al., 2002; Fahie-Wilson & Ahlquist, 2003). Macro-PRL is a macro-molecular complex of prolactin (PRL) with an IgG antibody (Schiettecatte et al., 2001, 2005) directed against specific epitope(s) on the PRL molecule. Macro-PRL is considered biologically inactive in vivo because of its decreased bioavailability. Macro-PRL is cleared more slowly than monomeric PRL and hence accumulates in the sera of affected subjects. The incidence of macro-PRL is up to 26% of all reported cases of hyperprolactinemia depending on the immunoassay system. Macro-PRL is detected in various degrees by different immunoassays (Smith et al., 2002). Laboratories should know the reactivity of the PRL assay with macro-PRL and ideally test for the presence of macro-PRL in all patients with hyperprolactinemia by gel filtration chromatography or pre-treatment with polyethylene glycol (PEG 6000). It is important to both recognize the presence of macro-PRL and provide an estimate of the monomeric PRL concentration because some patients with macroprolactinemia may have clinically significant, elevated monomeric PRL levels also (Van Besien et al., 2002; Fahie-Wilson, 2003).

5. Alteration of antibody binding

5.1 Heterophilic antibodies

Heterophilic antibodies are antibodies produced against poorly defined antigens. They are multi-specific antibodies of the early immune response and generally show low affinity and weak binding (Levinson & Miller, 2002). These antibodies react with many antigens and the variable region of other antibodies (anti-idiotypic antibodies). IgM antibodies play a key role in interfering sera from rheumatic patients as they can bind Fc fragments of human antibodies (Ismail et al., 2002b).

Interfering, endogenous antibodies should be called heterophilic when there is no history or medical treatment with animal immunoglobulins or other well-defined immunogens, and the interfering antibodies are multi-specific (reacts with immunoglobulin from two or more species) or exhibit rheumatoid activity (Kaplan & Levinson, 1999). In case of rheumatoid factor (RF), false elevated results arise from the binding of RF to the Fc constant domain of antigen-antibody complexes. The presences of RF in serum can cause falsely elevated analyte levels in troponin assays (Fitzmaurice et al., 1998), thyroid function tests (Martel et al., 2000), tumour marker assays (Berth et al., 2006) and falsely detected HCV-specific IgM (Stevenson et al., 1996).

In two-site IMA's, heterophilic antibodies can bridge two assay antibodies together and falsely elevates the patient value by producing an assay signal (Boscato & Stuart, 1986). Assays using either polyclonal or monoclonal antibodies can be affected. The same

heterophilic may react differently for different antibody combinations hence causing rise in one assay but a lower result in another assay. The presence of excess non-human immunoglobulin in the assay buffers reduces the possibility of the interfering substances binding to the capture and detection antibody by binding instead to the interfering immunoglobulin. Although manufacturers routinely add blocking agents to their assay formulations, not all heterophilic interference can be blocked by non-immune globulin, including pooled globulin from several species as heterophilic antibodies may show reactivity to idiotypes that are not present in the blocking reagent. Both IgG and IgM heterophilic antibodies are reported to occur (Covinsky et al., 2000).

5.2 Human anti-animal antibodies

Human anti-animal antibodies (HAAA) are high-affinity, specific polyclonal antibodies generated after contact with animal immunoglobulin. They show strong binding and are produced in a high titer. HAAAs can be of the IgG, IgA, IgM, or rarely, the IgE class (Kricka, 1999). They compete with the test antigen by cross-reacting with reagent antibody of the same species to produce a false signal. The most common HAAAs are human anti-mouse antibodies (HAMA), but also antibodies to rat, rabbit, goat, sheep, cow, pig, horse may occur (Selby, 1999). HAMA is especially prevalent in serum of animal workers and in patients on mouse monoclonal antibody for therapy or imaging.

Interfering, endogenous antibodies should be called specific HAAAs when there is a history of medical treatment with animal immunoglobulin and immunoglobulin from the same species used in the immunoassay (Kaplan & Levinson, 1999). The nomenclature becomes confusing where the immunogen is not known and a heterophilic antibody is recognized in mouse or other animal-specific immunoassays.

HAMA interference has been reported for numerous analytes including cardiac markers assays (White & Tideman, 2002), thyroid function tests (Frost et al, 1998), drugs and tumour markers (Boerman et al., 1990). Two-site (sandwich) immunoassays are more prone to interference from antibodies to animal IgG in serum and may cross-react with reagent antibodies especially from the same species. HAMAs interfere by bridging between the immunoglobulin capture and the immunoglobulin detection antibodies resulting in false-positive results. False negative results due to HAMA interference are also possible in two-site assays, when the HAMA reacts with one of the antibodies preventing reaction with the analyte (Kricka, 1999). Methods that use only one mouse monoclonal in IMA assays are less prone to interference from HAMA.

5.3 High-dose hook effect

The hook effect is based on the saturation curve of antibody with antigen (Figure 2). It is caused by excessively high concentrations of analyte simultaneously saturating both capture and detector antibodies. The high-dose hook effect occurs mostly (but not exclusively) in one-step immunometric (sandwich) assays, giving a decrease in signal at very high concentration of analyte (Fernando & Wilson, 1992). In immunoassays with very large analyte concentration ranges (ferritin, growth hormone, hCG, PRL, Tg, tumor markers PSA, CA19.9, CA125); antigen-antibody reactions can go into antigen excess and result in falsely decreased results and potential misdiagnosis. In one step two-site immunoassays where

capture and detection antibody are added simultaneously, free analyte and analyte bound to the labeled antibody compete for the limited number of antibody-binding sites of the detector and in the presence of very high analyte concentration will decrease in stead of increase label bound to the solid phase. High-dose hook effect can be avoided by increasing the quantity of the reagent antibodies and by reducing the amount sample required for analysis or by sample dilution (Cole, 2001). Careful assay design is necessary to ensure that the concentrations of both capture and detector antibodies are sufficiently high to cope with levels of analytes over the entire pathological range. It is common practice to re-assay samples at several dilutions as a check on the validity of the result (Davies, 2005).
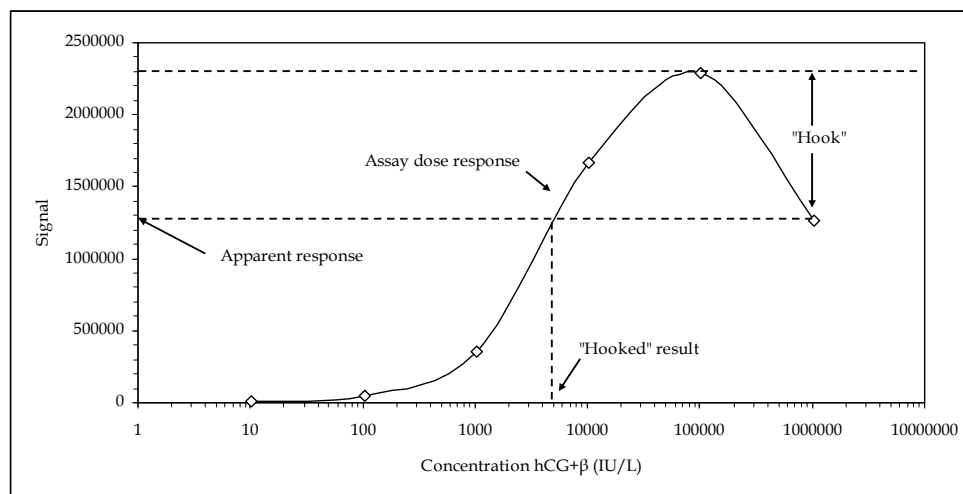

Fig. 2. High-dose hook effect in Elecsys hCG+β assay - an excessive amount of hCG overwhelms the binding capacity of the capture antibody. This results in an inappropriately low signal that causes an erroneous "hooked" result (6200 IU/L) for a patient with an excessively elevated serum hCG+ β concentration of 1 030 000 IU/L.

5.4 Other proteins

Interfering proteins of general relevance include albumin, complement, lysozyme, fibrinogen and paraprotein (Tate & Ward, 2004). They can affect antibody binding and can cause interference in immunoassays. Albumin may interfere as a result of its high concentration and its ability to bind or release large proportions of ligand. Complement binds to the Fc fragment of immunoglobulins and can block the analyte-specific binding sites of antibodies (Weber et al., 1990). Lysozyme can form a bridge between the solid-phase IgG and the detector antibody (Selby, 1999). IgG kappa paraprotein can bind to a TSH assay antibody and sterically block the binding of TSH and lead to falsely lowered TSH values (Luzzi et al., 2003).

6. Interference with detection systems

Occasionally, some samples contain compounds that artificially increase or decrease the magnitude of the response, without affecting antigen-antibody binding.

6.1 Endogenous signal-generating substances

The presence of endogenous signal-generating substances can interfere in the signal detection of an immunoassay. Diagnostic or therapeutic administration of radioisotopes can be carried over to the final counting tube, altering radioimmunoassay results. Endogenous europium can interfere in time-resolved fluorescence. With fluorescent immunoassays, interference can result from endogenous fluorescent substances, fluorescent drugs or fluorescein administration for the performance of retinal angiography (Davies, 2005).

6.2 Enzyme inhibitors/activators

In enzyme-labelled immunoassays, the presence of inhibitors or activators of the detection enzyme in the sample may alter the signal and thereby the immunoassay results. Enzyme inhibitors can be chemical or immunological. Antibodies that cross react with horse-radish peroxidase or alkaline phosphatase have been described. Azide present as preservative in some control sera may lead to suppression of enzyme activity in assays using peroxidase as label. Samples collected into tubes containing sodium fluoride may be unsuitable for some enzymatic immunoassay methods due to inhibition of the enzyme activity by fluoride (Davies, 2005).

6.3 Enzyme catalysts or cofactors

Enzyme-immunoassays can be affected by enzyme catalysts or cofactors, for example Cu^{2+} contamination promoting luminol chemiluminescence in the presence of H_2O_2.

Some label interferences can be resolved by utilising a heterogeneous assay format, a pre-treatment step, screening specimens for endogenous radioactivity before assay, use of non-isotopic labels/methods or diluting the sample so that the interfering substance is also diluted. These interferences can also selectively be depressed by adding suitable blocking agents (Davies, 2005).

7. Incidence of immunoassay interference

The prevalence of interference in modern immunoassays is low, but variable and dependent on the type of antibody interference. Heterophilic antibody and HAMA interference can vary from 0.05% to 6% depending upon the method of detection (Bjerner et al., 2002). Non-analyte antibody binding substances have been detected in proximally 40% of serum samples using a modified immunometric assay, termed an "interference assay" and they caused 15% interference in non-blocked assays (Boscato & Stuart, 1986). Ward et al. identified 7 out of 21,000 samples from a hospital population with heterophilic interference and HAMA, the interference being as low as 0.03% in blocked IMAs. However, the addition of blocking reagent does not guarantee the complete elimination of interference (Ward et al., 1997).

The extent of affected immunoassays was highlighted in a multicenter survey of erroneous immunoassay results from assays of 74 analytes in 10 donors conducted by 66 laboratories in seven countries (Marks, 2002). Approximately 6% of analytes gave falsely elevated results with the potential for incorrect clinical interpretation. Of these analytes, 1.8% (n=65) of

results involving 13 analytes were determined to be heterophilic false-positive while another 4.2% (n=146) of results involving 17 analytes gave false-positives of uncertain etiology that were not restored to within the reference interval by addition of heterophilic blocking reagent. The blood was obtained from donors with RF-positive illnesses, multiple sclerosis, or lupus, and had detectable RF (31 to >1000 kIU/L) and/or HAMA (3-589 µg/L). Blood from nine of the ten donors resulted in false-positive results of uncertain etiology for six of seven estradiol assay systems (58% of analyses performed) and for two of eight cortisol systems (20% of analyses). For blood from one donor, eight of eleven FSH and LH assay systems reported false-lowered results. The highest percentage of heterophilic false-positive results in this survey occurred for myoglobin (48% of analyses performed in two of seven tested assay systems). From the available evidence, Levinson & Miller assumed that the amount of interference identified with modern blocked two-site immunoassays is very low, in the order of 10 per 20000 samples assayed (Levinson & Miller, 2002).

8. Techniques to minimise antibody interferences in immunoassay

Methods for the reduction of heterophilic and anti-animal interference in immunoassays are summarized in Table 1 (Selby, 1999; Tate & Wald, 2004). These include ways to remove or block the interfering antibody (Kricka, 1999). Prior extraction of the analyte from the sample can remove the interference. Gel chromatography can be effective to remove interferants. Immunoextraction using murine monoclonal antibody or protein G immobilized on Sepharose beads has been effectively used to remove HAMA interferences. Anti-animal interference can also be removed by precipitation with polyethylene (PEG) 6000 (Ismail, 2005). Heat treatment (70-90°C) of samples is of limited utility because few analytes are heat-stable and thus do not survive these antibody-denaturing conditions.

Addition of low concentrations of serum or immunoglobulin from the same species as the antibody reagents in the reaction mixture can prevent interference in some samples by neutralizing or inhibiting the interference. The blocking agent can be included in the assay diluent or the sample can be pre-treated before assay. Non-immune serum, polyclonal IgG, polymerized IgG, non-immune mouse monoclonal, or fragments of IgG (Fc, Fab, F(ab')$_2$) from the same species used to raise the reagent antibodies, are commonly used as blocking agents. However, in some cases addition of one or more of these blocking agents in immunoassay reagents is either insufficient or not successful in preventing interference. Determination of the exact amount of blocker sufficient to eliminate interference in all patient samples is difficult to determine in practice as the immune response to interfering antibodies is highly variable between individuals. The effectiveness of the added blocking agent depends on the species and subclass of the blocker (Selby,1999; Kricka, 1999; Tate & Ward, 2004). Several blocking reagents are available commercially: Heterophilic Blocking Reagent (HBR; Scantibodies), Immunoglobulin Inhibiting Reagent (IIR; Bioreclamation), Heteroblock (Omega Biologicals), MAB33 and Poly MAB 33 (Roche Diagnostics).

Another solution for the problem of human-animal antibody interferences is the use of Fab or F(ab')$_2$ fragments instead of the intact immunoglobulin as capture or detector antibodies in two-site assay, eliminating the interference of HAAAs with specificity for the Fc portion of an IgG antibody. Another strategy is to use chimeric antibodies. These chimeric antibodies

are human antibodies where the variable regions are replaced with the corresponding part of a non-human antibody (mouse or rat). Interferences by anti-mouse or other animal antibodies are eliminated (Kricka, 1999). The latter are now used in some Roche immunoassays (Elecsys TSH, CEA, Troponin T) either as capture or detector antibody.

Removal of interfering antibody
• Extraction of analyte from sample
• Immunoextraction by addition of murine or other animal species serum immobilized onto Sepharose beads or immobilized Protein A suspension
• Polyethyleen glycol precipitation (PEG 6000)
• Heating to 70-90°C for heat-stable analytes
Addition of blocking agent from the same species as the antibody reagents
• The inclusion of one or more blocking agents in manufacturers' immunoassay reagent may be insufficient to overcome the interference
• Non-immune serum, species-specific polyclonal IgG, anti-human IgG or polymerized mouse IgG
• Non-immune mouse monoclonals
• Species-specific fragments of IgG (Fc, Fab)
• Heterophilic blocking reagents (HBR), immunoglobulin inhibiting reagent (IIR), and antibody blocking tubes
Assay redesign
• Use of Fab or F(ab')2 fragments
• Use of chimeric monoclonal antibodies

Table 1. Methods for reduction of interference from heterophilic antibodies and human anti-animal antibodies (Selby, 1999; Tate & Wald, 2004)

9. Testing for interferences in samples suspected of interference

Immunoassay results on samples suspected of interference can be checked by different procedures (Table 2). These include repeat analysis of the sample using a different immunoassay platform that, if possible, employs antibodies that are raised to a different species and normally gives agreement between methods. If HAMA interference is suspected, the alternate assays should not use monoclonal mouse antibodies because the assay may also be inaccurate. If a significantly different result is detected between methods there is a likelihood of interference. However, agreement between methods does not necessarily exclude interference nor does disagreement, if methods lack standardization and clinical decision limits differ (Tate & Wald, 2004). The false assumption that a result is correct because a majority of immunoassay methods give similar results was shown in the multicentre study by Marks in which nine of eleven LH and FSH methods were in agreement but gave falsely low results for a 72-year old postmenopausal woman who was positive for RF (Marks, 2002). Reanalysis using alternative technology such as liquid chromatography or tandem mass spectrometry should be considered if available (Ismael, 2009; Hoofnagle & Wener, 2009).

Another procedure for detecting and identifying a suspected interfering antibody is the use of commercially available blocking antibodies (Emerson et al., 2003). Statistically discrepant results before and after incubation with blocking agent would be indicative of interference. A difference between initial and treated value of 3 to 5 standard deviation (SD) suggest possible heterophilic interference, >5 SD indicates definite heterophilic interference (Preissner et al., 2005). However, 20-30% of samples with interfering antibodies may yield similar results after treatment with the blocking antibodies (Ismael, 2009).

Another test is making serial dilutions of the sample using manufacturer's diluent, provided that it contains non-immune globulin (Ismail, 2007). This could identify about 60% of samples with interference in which linearity and parallelism are lacking.

Using these three tests could identify interference in almost 90% of suspected samples (Ismail, 2009).

Repeat analysis with an alternate immunoassay that preferably uses antibody raised to a different species or using alternative technology such as liquid chromatography or tandem mass spectrometry
Measurement before and after addition of a blocking reagent
Measurement of dilutions of the sample with the manufacturer's diluent containing non-immune globulins
Sample pre-treatment e.g. PEG precipitation in Prolactin measurement

Table 2. Methods for testing of interference in suspected samples (Tate & Wald, 2004)

10. Conclusions

Interference in immunoassays from endogenous antibodies is still a major unresolved and underestimated analytical problem, which can have important clinical consequences. There is no single procedure that can rule out all interferences. It is important to recognize the potential for interference in immunoassay and to put procedures in place to identify them wherever possible. Most important is a consideration of the clinical picture. If there is any suspicion of discordance between the clinical and the laboratory data an attempt should be made to reconcile the difference. The detection of interference may require the use of another method, or measurement before and after treatment with additional blocking agent, or following dilution of the sample in non-immune serum. If testing is inconclusive and the interference cannot be identified, the analyte concentration should not be reported and laboratory report should indicate there is a discrepancy for that analyte due to some technical inaccuracy and suggest the test to be repeated using another sample.

Interference in immunoassay is one factor that contributes to the uncertainty of medical testing. Laboratories should be aware of the potential for interference in all immunoassays and how artefactual results may cause misinterpretation and a subsequent erroneous diagnosis and unwarranted treatment. The recognition of such aberrant test results requires constant surveillance of both laboratory and clinician. Since these interferences are relative uncommon, clinicians need to be aware of them and alert to the mismatch of clinical and

biological data. Dialogue between the clinician and the clinical laboratory over unexpected immunoassay test results can avoid inappropriate clinical intervention based on abnormal test results.

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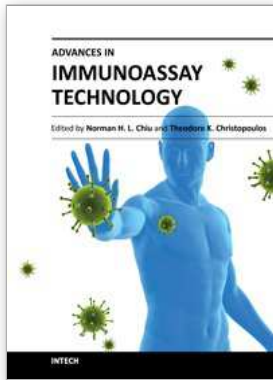
Advances in Immunoassay Technology

Edited by Dr. Norman H. L. Chiu

From the basic in vitro study of a specific biomolecule to the diagnosis or prognosis of a specific disease, one of the most widely used technology is immunoassays. By using a specific antibody to recognize the biomolecule of interest, relatively high specificity can be achieved by immunoassays, such that complex biofluids (e.g. serum, urine, etc.) can be analyzed directly. In addition to the binding specificity, the other key features of immunoassays include relatively high sensitivity for the detection of antibody-antigen complexes, and a wide dynamic range for quantitation. Over the past decade, the development and applications of immunoassays have continued to grow exponentially. This book focuses on some of the latest technologies for the development of new immunoassays.

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