ASHI-U Module

Title: Serum Preparation

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Section 2: Objectives

- To understand the clinical rationale for using different types of serum preparations for antibody screening and/or crossmatching.

- To understand the limitations of and quality control necessary for different types of serum preparations.

- To understand the pros and cons of different types of serum preparations.

- To understand the ASHI Standards that are applicable to serum preparation.
Section 3: Introduction

A critical component of testing in the HLA laboratory is the ability to detect antibodies in the patient serum. This can be accomplished by antibody screening and identification or crossmatch techniques. IgG antibodies directed against foreign HLA molecules are considered to be the most clinically relevant class of antibody. Whatever the method used, it is important for laboratories to be able to process patient serum samples in such a way to minimize background and/or non-specific reactivity. It is common for patient serum to contain non-clinically relevant antibodies, complexes, or contaminating substances that interfere with the detection of clinically relevant antibodies.

The methods discussed will be re-calcification of plasma, absorption with lymphocytes, inactivation of IgM antibodies by DTT and heat inactivation, depletion of OKT3 from serum, and general concepts in preparation of monoclonal antibodies. Each method section will contain instructions on specimens, instrumentation, reagents, procedure, and troubleshooting. All methods described in this document should be validated before putting into use.

Objectives of this will be: 1) to understand different types of serum preparation and their uses, 2) to outline a protocol and understand the quality control necessary for different types of serum preparation, and 3) to weigh the pros and cons of each type of serum preparation and understand the ASHI standards that are applicable to each method.
Section 4: Methods and Instrumentation

Re-calcification of Plasma

In most assays, serum is recommended over plasma because the anticoagulant may interfere with testing. Plasma converted to serum is useful as a typing reagent, because it is usually collected in large volumes from whole blood donors or plasmapheresis. This is achieved by adding calcium to plasma to form a complex with the citrate. This binding restores the normal ionized calcium level allowing the sample to clot. The optimal amount of calcium needed to convert plasma to serum varies depending on the volume of blood, calcium binding capacity of plasma proteins and other anions, and the donor’s hematocrit. However, the required amount of calcium to ensure complete clotting, given sufficient time, does not have to be exactly optimal. Arbitrary addition of average amount of calcium required to restore a normal ionized calcium level almost always has the desired results.

Absorption with Lymphocytes and Platelets

Testing serum for antibodies allows clinicians to know what organs are unacceptable. These antibodies may be tested by Cytotoxicity, Flow Cytometry, or Microarray. Multispecific serum can be problematic when trying to determine the specificities using cells or phenotypic antigen coated beads. Serum can be absorbed with lymphocytes to determine if it is a mixture of antibodies or a single antibody. For example, a serum that reacts with A1 and B8 can be absorbed with lymphocytes expressing A1 but not B8. The absorbed serum will be retested against B8 to see if there is still reactivity or if the reactivity is only directed against A1. This technique allows for the selective removal of antibodies.

Another application of absorption with lymphocytes is to remove autoantibodies. These autoantibodies may be cold or warm reacting, and may complicate the interpretation of crossmatch tests. Autoantibodies may cause false positive crossmatches or hinder the characterization of alloantibodies.
Inactivation of IgM Antibodies by DTT Treatment and Heat Inactivation

Antibodies directed against HLA antigens are typically IgG isotype and antibodies directed against self-antigens are typically IgM isotype. IgM antibodies are common in patients whose renal failure is caused by systemic lupus erythematosus. These antibodies can bind to reagent, patient, or donor cells, activate complement and become detectable in antibody screens and crossmatches. For this reason, IgM antibodies should be inactivated to reduce the chances of false positive tests. Inactivation is achieved by two methods: DTT treatment and heat inactivation.

DTT (dithiothreitol or Cleland’s reagent) is a sulfhydryl compound which inactivates IgM antibodies by cleavage of the intersubunit disulphide bonds. IgG antibodies are less susceptible to inactivation by DTT because the disulfide bonds between chains are not as labile as the disulfide bonds between the IgM subunits but they may be slightly affected. DTT inactivation can be carried out directly in microtiter plate wells or tubes. DTT can adversely affect the function of complement. To avoid this, add cysteine to the complement or perform two additional washes before adding complement to the wells. DTT that is prepared in PBS should be stored at -20°C, while DTT prepared in isotonic saline should be stored at 4°C.

Heat inactivation works by denaturing the heat sensitive proteins. This technique is less specific than DTT, but IgG molecules are relatively insensitive to heat and are minimally affected compared to IgM molecules. It has become a popular choice due to speed, lack of sample dilution, and absence of carcinogenic chemicals. However, denatured IgM antibodies may interfere with platelet absorption and therefore, absorb serum with platelets before DTT treating the serum. Heat inactivation is not usually performed on bone marrow transplant patients. Antibody screening is performed on patients who do not achieve the expected increase in platelet count from random donor platelet units. IgG HLA antibodies directed against the HLA antigens of the platelet donor are a
common cause of platelet refractoriness. However, IgM antibodies found in these patients are usually newly formed alloantibodies, and not autoantibodies.

**Depletion of Therapeutic Anti-Lymphocyte Antibodies from Serum**

Transplant recipients may be given therapeutic anti-lymphocyte antibodies to help aid in the reversal of acute rejection. These therapeutic antibodies may cause a positive reaction in the T-cell lymphocytotoxicity assay. This positive reaction may mask an underlying alloantibody and therefore, the therapeutic anti-lymphocyte antibodies should be adsorbed out before testing. Murine therapeutic antibodies such as OKT3 may be absorbed with a magnetic bead coated with sheep anti-mouse immunoglobulin. Multiple absorptions may be needed for complete removal of OKT3 if the concentration is more than 1000 ng/ml. If serum is still reactive after multiple absorptions, a true antibody may have been identified. These magnetic beads can be washed by utilizing a magnet or centrifugation. OKT3 is usually given daily 10-14 days after diagnosis of acute rejection. Trough levels increase in the first two days and then reach a steady state of approximately 900 ng/ml for the remainder of the treatment course. OKT3 is cleared from plasma within 48-72 hours after final dose.
Section 5: Link to ASHI Lab Manual
Section 6: Frequently Asked Questions

**Question:** Why is calcium added to plasma to convert it to serum?
**Answer:** Calcium is added to the plasma to combine with the citrate present in the anticoagulant. This restores a normal ionized calcium level and allows clotting to occur. Once a clot has formed, the recalcified plasma can be collected and used for testing.

**Question:** At what temperature should serum absorption with lymphocytes be performed?
**Answer:** Typically, auto-antibodies are cold reacting and should be absorbed at 4°C while alloantibodies are warm reacting and should be absorbed at 37°C.

**Question:** Which serum treatment should be used to remove IgM antibodies, DTT or heat inactivation?
**Answer:** The specific type of serum treatment used depends on the agreement between your program’s clinicians and your laboratory. DTT treatment is more specific than heat treatment in removing IgM antibodies. However, DTT can also affect the disulfide bonds in IgG antibodies. It is important to run all of the controls described to ensure that either treatment has only a minimal effect on IgG and does not inhibit complement.

**Question:** Why are so many controls used when treating serum with DTT?
**Answer:** The controls are necessary when using treated serum to ensure that treatment is denaturing the IgM antibody and not affecting the IgG antibody. Additionally, the dilution control used with DTT treatment ensures that any diminished reactivity is not due to the dilution of serum with DTT.

**Question:** What happens if we don't remove OKT3 prior to cytotoxicity assays?
**Answer:** OKT3 is a monoclonal antibody directed against the CD3 molecule. Therefore, the binding of OKT3 to any CD3+ cell will activate complement and lyse the target cell. This results in false positive reactivity.
Section 7: ASHI Standards

Revisions to the ASHI standards may have taken place since the development of this document. Laboratories and individuals should always reference the most current ASHI approved standards.

Section 8: References


