THE INDIAN IMMUNOHEMATOLOGY INITIATIVE WET WORKSHOP "SOP";
A Comprehensive, One Week Introduction to Blood Group Antibody Identification

Jim Perkins, MD, Martha Rae Combs, MT(ASCP)SBB,
Janis Hamilton, MS, MT(ASCP)SBB, Susan Johnson, MSTM, MT(ASCP)SBB^{CM}

INTRODUCTION

This document presents the procedure for the standard week-long wet workshop developed by the Indian Immunohematology Initiative and used over a dozen times over as many year with continuous and ongoing refinement. Our goal in developing this curriculum was to train participants to perform the most important immunohematology (IH) procedures needed to handle the majority of patient compatibility problems, and our goal in publishing this "SOP" is to encourage others to embark on hands-on IH training where appropriate. The curriculum objectives are covered in a set of 5 cases of increasing complexity, plus an introductory skills exercise, a daily QC procedure and a serologic centrifuge calibration exercise if time permits. The exercises are interspersed with short lectures covering the theoretical basis of the testing performed (see workshop schedule, appendix #1). Finally, if scheduling permits, on the Saturday after the workshop multiple paper cases are presented with the objectives of reinforcing and extending the participants' new cognitive skills in analyzing IH cases, and of engagement with the local immunohematology community. If less than a week is available for the workshop some subset of the exercises can be performed.

All workshop testing is performed by "tube agglutination". Although more and more laboratories in India and other parts of South Asia are acquiring the capability to perform testing by other manual and automated methods, many more laboratories cannot afford these technologies. Moreover, we believe that to perform at the highest level a laboratory must retain the ability to perform tube tests for special procedures and for problem resolution when sensitive testing formats such as column agglutination (gel or beads) and solid phase yield non-specific reaction patterns. So enhancing the participants' tube testing skills is one objective of the workshop. The cognitive skills required for serologic problem resolution are identical for all testing platforms, so the analysis applied to the exercises is applicable to laboratories using other platforms for their primary test method.

Our reliance on tube agglutination makes it preferable that each student have access to a serologic centrifuge, and if this model is followed the number of available centrifuges (and incubators) will determine the class size. The workshop can easily be performed with two students sharing a 12-place centrifuge, and although this slows the pace somewhat it is still possible to complete all 6 exercises within the 5-day format. Of note however, if testing were performed by the gel method, potentially the class could share a single centrifuge and incubator.

A laptop computer and projector are also required for the workshop. This can be set up in a room separate from the testing room, but the class will go faster if the students do not need to leave their seats for the short lectures. It is also essential to have either an erasable white board, a blackboard or an easel with a large paper flip board in order to write procedures and notes for each exercise for the participants and to expand on the lecture content. Finally, it is very useful to have an overhead projector or "document" camera so the instructor can record students' results on a facsimile of their worksheets. These results can then be discussed and analyzed by the instructor and class together. It also allows the students to see how their reading of agglutination compares to others in the class. If an overhead projector is used the worksheets must be copied onto transparency sheets. More convenient is a document camera a small camera on a stand that can be connected to the computer projector and which projects the paper worksheet below it (e.g. Elmo^{TM}).

Workshop participants receive a workbook that includes the schedule, worksheets for recording the results of each exercise including antigen profiles for the cell panels and the QC worksheet, a short manual of abbreviated instructions for the serologic tests required in the exercises, and blank pages on which the participants can take notes. For some workshops we have also provided a workbook of serologic case studies.

Specimens, reagents, materials, and equipment used in the workshop are listed in the spreadsheet in appendix #2 by exercise and summed for each item. When the identity of the specimens and the number of participants is entered into the spreadsheet it will calculate many of the amounts of reagents and materials needed.

If this workshop plan isn't used in its entirety it may at least provide a starting place for individuals planning to give similar workshops. In particular workshop instructors would be expected to customize the lectures or replace them with their own materials. Like other educational materials on the III website, this curriculum and associated documents are intended for use by other educators. Therefore permission to use this material is automatically granted. Nonetheless, when these materials are used all or in part by others the III would appreciate being credited. Others should not present items substantially similar to these provided as entirely their own intellectual property.

Premises of the III teaching program:

- Case-based training works best to establish both theoretical and practical understanding.
- Hands-on or "wet" training is the most effective way to teach techniques of blood group antibody detection and identification. Lectures work best in association with hands-on teaching.
- Tube agglutination testing offers the best combination of availability, accuracy, and expense for resource-limited blood banks. Moreover, advanced immunohematology testing requires proficiency with tube methods even when non-tube and automated methods are available.

INTRODUCTORY SKILLS EXERCISE: Prepare a 3% RBC suspension and grade agglutination reactions

Objectives

At the end of the exercise participants will be able to:

1. Prepare an accurate 3% RBC suspension (some variation is acceptable);
2. Grade tube agglutination reactions accurately;
3. Utilize workshop equipment and materials appropriately;
4. Follow good laboratory and testing practices.

Summary

In this exercise the participants prepare a 3% suspension of group A RBCs in saline.

Before class the instructors prepare a two-fold dilution of group O donor plasma. Each student tests their RBC suspension against the dilutions by "immediate spin" (IS) testing. Each student's results are recorded so that they can be compared. Testing is then repeated with the same dilution and discussed with the hope that reaction reading will be somewhat more uniform the second time.

Lecture

The first lecture (see slide presentation appendix #4a) is presented before the exercise. It introduces the scope of immunohematology, discusses the importance of a 3% RBC suspension, illustrates reaction grading, outlines some important good testing practices and good laboratory practices, and gives instructions for the exercise.

In addition Appendix #7 includes video demonstrations of reading reaction strengths in tubes that can be used during the discussion of technique.

Materials, specimens, and specimen preparation specific to the exercise

1. Group A (preferably A1 lectin negative) donor RBCs: one tube for each student, labeled.
2. Serial dilution of group O donor plasma $\geq$ 8 drops per student. (Dilution should be carried far enough out so that at least 2 dilutions are nonreactive.)

Procedure (all procedures are performed according to the workbook procedures including attention to good laboratory practices)

1. Participants prepare a 3% cell suspension. Instructor(s) provide individual critique.
2. Participants test the group O plasma dilutions against their group A RBC suspension by the IS tube agglutination test, recording results in their workbook (see appendix #3).
3. Instructors verbally collect results (record the # of students with a 4+ rxn, # with a 3+ rxn, etc. on an overhead projector transparency or white board, etc.) and discuss as a group.
4. Repeat the exercise, collect results and review the spread of results between participants.

Comments
In spite of the fact that workshop participants, both physicians and technical personnel, are expected to have IH experience, in fact they typically arrive with varying manual testing skill levels. We also note that even seemingly experienced participants learn something from this initial exercise. The exercise focuses on the most basic technical issues in isolation at the start of the workshop so the class can function more as a group on later exercises. Improvement in agglutination grading skills may be seen as a decrease in the spread of the participants grading results between the first and second round of testing. The higher the titer of the anti-A the more tests the students will need to do and the greater the range of reaction strengths they will be able to observe.

Good laboratory practices and technical details emphasized in the lecture and exercise include:

- Use of serologic centrifuges;
- Unequivocal labeling of tubes;
- Use of a uniform drop size by holding the pipette at a uniform 45^{o} angle;
- Adding the plasma before the RBCs in tube testing;
- Mixing all RBC suspensions before filling the dropper;
- Reading of the most dilute sample first;
- Turning the tube so that the cell button is up and then gently resuspending it;
- Use of a viewing mirror to watch the button as it is suspended;
- Immediate recording of all reactions after reading.

Instructors should move around the classroom during each exercise watching and correcting the above throughout the workshop. Correcting student's technique for resuspending the cell button is particularly important, and concurrent documentation of results must also be repeatedly emphasized.

In preparing RBC suspensions some variation in concentration is inevitable; it's better for RBC suspensions to be on the heavy than the light side since inexperienced individuals may lose RBCs during wash steps. The students can compare their suspensions to the commercial cell reagents. Although addressed in the lecture, the impact of RBC suspensions that are too light or too heavy must be emphasized throughout the exercise.

This exercise should not be rushed. Additional time that can be spent in repeated practice will have benefits in the later cases.

In addition to testing the sequential dilutions twice, the instructors might make a series of 10 to 12 samples with known dilutions covering the ranges of reaction strengths but that are out of sequential order. This will aid the students in reading actual unknown samples when the reaction result cannot be anticipated.

Note regarding samples for Cases 1-3 and Case 5; Alternate materials or "faking it"
Strong antibody-containing plasmas are hard to come by, although anti-D, anti-E, and anti-K used in Case #1 are somewhat easier. It is possible to mimic the intended antibody reactivity pattern in Cases 1-3 by using plasma containing anti-D (possibly including diluted Rh immune globulin (RhIG) and using a created antibody identification panel where the appropriate panel cells have been replaced with 3% suspensions of RhD-pos or RhD-neg donor RBCs in the desired pattern of reactivity. This requires careful planning on the part of the instructors to have specific panels for each of the exercises but greatly simplifies the sample acquisition process. The warm autoantibody in Case 5 has been performed using anti-D in all but one of the workshops to date as a true warm autoantibody is nearly impossible to find in sufficient quantity Additional notes for the alternate materials can be found in each case if appropriate.

CASE #1; Type-and-screen, identification of a simple, single antibody specificity, and selection of compatible donor RBCs

Objectives
At the end of the exercise participants will be able to:

1. Perform and interpret a type-and-screen (T&S);
2. Identify the importance of IgG sensitized (Coombs) control cells ("CCC", "CC" or "check cells");
3. Describe the difference between RBCs expressing a blood group antigen in single versus double dose;
4. Systematically analyze a single, simple antibody, and state the criteria for proof of the antibody specificity;
5. Perform and interpret crossmatches performed by the indirect antiglobulin test (IAT);
6. Perform antigen typing, including selection of appropriate positive and negative control cells;
7. Accurately prepare cell suspensions, grade reactions, and follow good testing practices.

Summary
In this exercise the participants perform a type-and-screen on a specimen from a patient with a single antibody. The antibody is identified including the patient's antigen typing. Three donor RBC specimens are provided and typed for the corresponding antigen as well as crossmatched. One donor is compatible, one is incompatible due to mislabeling with an incorrect ABO group, and one has a positive DAT causing an incompatible crossmatch.

Lectures

The second lecture "The Indirect Antiglobulin Test" (IAT, see slide presentation appendix #4b) is presented before the exercise. It reviews antihuman globulin and the direct and indirect antiglobulin tests including the importance of check cells. The IAT is discussed as a *test method* that can be used to detect and identify antibodies, type for blood group antigens, and perform a crossmatch. Modulators of sensitization and agglutination are discussed including incubation time and temperature, serum/cell ratio, albumin, LISS, PEG, enzyme treatment, and the dosage effect. Finally, the principles of column agglutination and solid phase adherence testing are briefly reviewed.

The third lecture "Antibody Identification" (appendix #4c) is given after the participants see and result a positive antibody detection test ("antibody screen") and before testing a panel. The "cross-out method' of panel analysis is introduced which prompts review of single- vs. double-dose expression of blood group antigens and exceptions to the rule of crossing out on cells with double dose expression (anti-K and anti-E and -C in the presence of anti-D). These are expanded on in the lecture #4. Finally the criteria for proof of identification of an alloantibody specificity are introduced.

Specimens and materials specific to the exercise (Reagents used in all exercises are listed at the end.)
1. Patient plasma containing anti-K, anti-E, anti-C or anti-D.
2. Patient RBCs of the same blood group as the plasma and lacking the K/E/C/D antigen as appropriate.
3. Three sets of donor RBC tubing segments from available donor units as follows:
   A. ABO compatible RBCs lacking the antigen corresponding to the case #1 antibody,
   B. ABO incompatible RBCs lacking the antigen corresponding to the case #1 antibody,
   C. ABO compatible RBCs lacking the antigen corresponding to the case #1 antibody but with a positive DAT (D-positive RBCs sensitized with anti-D from RhIG).
4. Eleven cell antibody identification panel.
5. Antigen typing sera for the antigen corresponding to antibody in the case #1 patient specimen.

Procedures
Participants perform each of the following tests. Instructors circulate through the class commenting on students' technique and answering questions. After each test instructors tabulate and analyze the results with the class. After each test instructors tabulate the participants' results on a transparency or paper worksheet under a document camera) and analyze the results with the class.

1. Perform a type-and-screen on a patient with anti-K, -E, -C or -D.
2. Test the patient plasma against an antibody identification panel including an autocontrol.
3. Type the patient and donor RBCs for the K, E, or C antigen.
4. Crossmatch the 3 donor RBC samples with patient plasma (2 are expected to be positive.)
5. Perform a DAT on the E-negative, crossmatch positive donor sample.

Comments
This exercise introduces pretransfusion testing routines typically used in the United States and other Western countries. For many of our students the exercise has been their first introduction to the systematic analysis of antibody identification procedures. Also for many students the exercise will also introduce the concept of T&S pretransfusion testing. Much time is devoted to discussion of the concept of 'ruling out' to identify the antibody and recognition of the dosage of antigens on the panel cells.

Technical details should be addressed with the group and with individual participants throughout the exercise including those listed for the introductory exercise plus:

- Blotting the last wash to achieve a dry cell button before adding AHG.

Students often avoid recording their reaction results in pen in the appropriate spot on the worksheet, instead waiting to record the "right" answer when it is revealed. Instructors must constantly be looking for this behavior and correcting it.

Antigen typing in this exercise can also be faked by using anti-D in a bottle labeled with the intended specificity as long as the patient RBCs used in the case are RhD negative. C protocol for the routine immunohematology reagents used in the laboratory.

DAILY REAGENT QUALITY CONTROL EXERCISE

Objectives
At the end of the exercise participants will be able to:

1. Analyze the utility of the various QC procedures performed;
2. Create a protocol for the routine immunohematology reagents used in the laboratory

Summary
In this exercise the participants perform daily QC of their standard reagent "racks".

Lecture/discussion
Rather than start this exercise with a prepared lecture the instructor(s) and participants discuss the rational for the tests outlined on the workbook QC sheet (included in appendix 3a) with reference to use a standard reagent "rack" as a prop. For example, the instructor might display the first reagent, and ask the class "What QC tests shall we do on this reagent?" If the first reagent selected were anti-A, the instructor would guide the class to selecting a test with the group A reagent cells as a positive control and with the group B reagent cells as a negative control. Then they would point out that the positive reaction of anti-A with A cells also serves as a positive control for expression of A antigen on the A cells and the lack of agglutination with the B cells demonstrates that they are not spontaneously agglutinating. The instructor and class then have the same discussion for each reagent in the standard rack, emphasizing how one test can QC multiple reagents including reagent RBCs, AHG and check Cells (CC). If the QC worksheet is projected with an overhead projector or computer document camera, the instructor can mask it with a piece of opaque paper and gradually reveal the various QC tests as they are discussed. The reagent QC antibody is introduced as a positive control for antigen expression on the antibody screening cells with the indirect antiglobulin test and demonstrates activity of the antihuman globulin. No reactivity at the IS phase demonstrates that the screening cell fail to agglutinate spontaneously.

Specimens and materials specific to the exercise
QC antibody reactive with all 3 antibody screening cells in the antiglobulin (AHG) phase. This can be a single antibody directed against a high-prevalence antigen such as anti-k or a mixture of antibodies generally commercially prepared.

Procedures
1. Reaction of reagent Anti-A with A and B reagent RBCs by "immediate spin" (IS, tubes #1 & 2),
2. Reaction of reagent Anti-B with A and B reagent RBCs by IS (tubes #3 & 4),
3. Reaction of reagent Anti-D with an Rh positive (RhD+) antibody screening (ASI or II) cell by IS (tube #5),
4. Reaction of reagent Anti-D with an Rh negative (RhD-) AS cell (ASIII) by IS and AHG phase testing (tube #6),
5. Reaction of the QC antibody with all three screening cells by IS and AHG phase testing (tubes #7, 8, and 9).

Comments
In the interest of time, daily QC of the serologic reagents is only performed on the second day. The QC procedures as listed on the III QC worksheet may not be the same as those used in participant labs; additional QC tests may be required by local regulations. Any differences should be discussed with respect to their utility. In our experience the discussion often turns to daily QC procedures vs. testing performed on new lots of reagents, and the difference must be discussed.

Good laboratory practice points to emphasize include:

- Recording of reagent lot numbers
- Recording expiration dates (do not use expired reagents)
- Signatures/initials of person performing testing

CASE #2; Identification of a single antibody specificity requiring selected cells

Objectives

At the end of the exercise participants will be able to:

1. Analyze antibody identification procedures for a single antibody, and state the criteria for proof of the antibody specificity;
2. Select and test rule-out cells needed for antibody identification;
3. Practice identifying the difference between single versus double dose antigen expression as well as the limits in selection of double dose rule out cells;
4. Perform an indirect antiglobulin test and antigen typing including control cell selection;
5. Practice preparing cell suspensions, grade reactions, and follow good testing practices.

Summary

In this exercise participants start directly with an antibody ID panel on a specimen from a patient with a single antibody that reacts with a sufficient number of panel cells that additional rule out cells need to be selected to complete (e.g. anti-c or anti-e). Patient antigen typing is performed to confirm the ID.

Lectures

The fourth lecture, "Rule Out Cell Exceptions" (appendix #4d) reviews the criteria for proof of an antibody specificity and then focuses on the requirement to rule out other common antibodies and the concept of single and double dose antigen expression. The lecture then looks at the limitations on selection of 'double-dose-expression RBCs', introducing the common Rh allele combinations, the concept of linkage disequilibrium, and how that limits availability of double-dose rule out cells.

Specimens and materials specific to the exercise

1. Patient plasma containing anti-c, anti-e, or some other antibody whose identification cannot be completed using a standard 11-cell panel, (i.e. selected cells are required).
2. Patient RBCs of the same blood group as the plasma and lacking the c or other antigen as appropriate.
3. Eleven cell antibody identification panel, including enzyme treated cell set if possible.
4. Second antibody confirmation panel (selected cells).
5. Antigen typing sera for the antigen corresponding to antibody in the case.

Procedures

Participants perform each of the following tests. Instructors circulate through the class commenting on students' technique and answering questions. After each test instructors tabulate and analyze the results with the class.

1. Test the patient plasma against an antibody identification panel (including an autocontrol).
   NOTE: No type-and-screen is performed in the interest of time.
2. Test additional panel RBCs to rule out antibodies not ruled out by the initial panel.
3. Type the patient RBCs for the proposed antigen specificity.

Comments

In order to save time this case study does NOT start with a T&S.

We have often found that an anti-c brought for this exercise that reacted well in the U.S. has become very weak by the time we have travelled to India. Therefore it is useful to have the option of using PEG enhancement or enzyme-treated panel cells.

As anti-c is a relatively common antibody in India, therefore it may be possible to locate a source in country.

If this case is performed using anti-D to mimic the intended antibody, the selected cell panels must also be prepared specific for the case.

CASE #3; Identification of multiple antibody specificities requiring selected cells

Objectives

At the end of the exercise participants will be able to:

1. Analyze antibody identification procedures to recognize and identify the presence of multiple antibodies, and meet the criteria for proof of each antibody;
2. Select rule-out and rule-in cells needed for identification of multiple antibodies;
3. Practice identifying the difference between single versus double dose antigen expression;
4. Practice performing an indirect antiglobulin test and antigen typing including control cell selection;
5. Practice preparing cell suspensions, grade reactions, and follow good testing practices.

Summary

As in the previous case this exercise start directly with an antibody ID panel on a patient specimen with multiple antibodies that requires selection of both rule-out and rule-in cells to complete. Patient antigen typing is performed to confirm the antibodies identified.

Specimens and materials specific to the exercise

1. Patient plasma containing multiple antibodies (e.g. anti-Fy^a + anti-S).
2. Patient RBCs of the same blood group as the plasma and lacking antigens as appropriate.
3. Eleven cell antibody identification panel, including an enzyme treated cell set if possible.
4. Second antibody confirmation panel (selected cells).
5. Antigen typing sera for the antigens corresponding to antibodies in the patient specimen.

Procedures

Participants perform each of the following tests. Instructors circulate through the class commenting on students' technique and answering questions. After each test instructors tabulate and analyze the results with the class.

1. Test the patient plasma against an antibody identification panel (including an autocontrol).
   NOTE: No type-and-screen is performed in the interest of time.
2. Test additional panel RBCs to rule-out and rule-in antibodies as needed;
3. Type the 'patient's' RBCs for the antigens corresponding to the hypothesized antibody specificities.

Comments

A point that often must be made clear in this case is that a rule-in cell for any of the antibodies can express only one of the blood group specificities thought to be present. On the other hand an antibody exclusion cell can rule out more than one specificity if it is non-reactive.

CASE #4; Investigation of an ABO discrepancy

Objectives

At the end of the exercise participants will be able to:

1. Systematically analyze a case of an ABO typing discrepancy, and plan further testing to resolve the discrepancy.
2. Practice identifying a blood group antibody including the necessary testing skills and selection of the rule-out and rule-in cells needed for identification of the cold alloantibody if used.

Summary

In this exercise the participants perform a type-and-screen on a specimen from a "patient" with an ABO discrepancy, preferably due to a single IS-reactive antibody. The antibody is identified including the patient's antigen typing, and the discrepancy is resolved by testing reverse typing cells lacking the responsible antigen.

Lectures

The fifth lecture, "ABO Discrepancies" (see appendix #4e) is given after the type-and-screen reveals the patient to have an ABO typing discrepancy and introduces the approach to such a problem through discussion of multiple simple cases.

Specimens and materials specific to the exercise

1. Patient plasma containing anti-M or anti-P1 reacting strongly at immediate spin. This problem can also be adjusted to use a strong cold agglutinin which may be more available. It cannot be faked with an AHG reactive anti-D but would if a large amount of reagent anti-D were available. It might also be faked with donor RBCs and plasma of different blood groups.
2. Patient RBCs of the same blood group as the plasma and lacking the M or P1 antigen as appropriate
3. Eleven cell antibody identification panel, including an enzyme treated cell set if the patient antibody is anti-M.
4. Second antibody confirmation panel, particularly if the patient antibody is anti-M.
5. Antigen typing sera for the antigens corresponding to antibodies in the 4th patient specimen.
6. Group A and B reverse typing cells lacking the M or P1 antigen as appropriate. If a cold agglutinin is used, the discrepancy may be able to be resolved using a pre-warmed technique.

Procedures

Participants perform each of the following tests. Instructors circulate through the class commenting on students' technique and answering questions. After each test instructors tabulate the results and analyze them with the class.

1. Perform a T&S on a patient plasma/serum specimen with anti-M or -P1.
2. Test the patient plasma against an antibody identification panel including an autocontrol.
3. Select and test rule-out and rule-in cells needed for identification of the antibody.
4. Type the patient RBCs for the hypothesized antigen specificity.
5. Resolve the ABO discrepancy by testing the patient serum against antigen negative reverse typing cells.
6. Perform a prewarmed test to determine the antibody significance if a cold alloantibody is the cause of the discrepancy.

Comments

This case works best with a strong anti-M. A strong cold autoantibody in a group A or B patient can be used for this exercise if a suitable alloantibody is unavailable.

In addition to presenting an ABO discrepancy, this exercise presents an additional opportunity to practice antibody identification. In that regard, since M and P1 are relatively high frequency antigens, after the first panel is run both rule-in and rule-out cells may need to be selected. If sufficient rule-out cells can't be found on the panels and the antibody is anti-M, other antibodies can be ruled out using enzyme-treated panel cells.

CASE #5; Investigation of a warm-reactive autoantibody

Objectives

At the end of the exercise participants will be able to:

1. Analyze antibody identification procedures to recognize and identify the presence of a warm-reactive autoantibody.
2. Perform a direct antiglobulin test (DAT).
3. Prepare and test an eluate.
4. Perform a warm autoantibody adsorption procedure, including treatment of the adsorbing cells, and describe the purpose of such a procedure.
5. Practice preparation of cell suspensions, grade reactions, and follow good testing practices.

Summary

In this exercise the participants perform a type-and-screen on a patient with a warm autoantibody causing a positive DAT and a panagglutinin in an antibody identification panel. An eluate is prepared and tested, and a warm autoadsorption is performed. The autoantibody in the "patient plasma" is mimicked by anti-D from a blood donor, and the antibody screening and panel cells are switched so all are RhD-positive, producing the appearance of a panagglutinin. The initial "patient RBCs" are Rh positive donor cells coated with RhIG producing a positive DAT. An eluate prepared from these cells produces a panagglutinin appearance with all RhD-positive screening or panel cells. The autoadsorption can be performed with RhD-positive "patient cells" which have not been coated with RhIG. To perform the adsorption the patient is “redrawn” and the additional RBCs provided are "switched" with the previous coated cells; when the adsorbing cells are not coated typically only a single adsorption needs to be performed.

Alternatively, the patient plasma (anti-D) can be adsorbed against Rh negative "patient RBCs" (switched for the initial "patient sample" of coated RBCs) and tested against a panel of D-positive and -negative RBCs created so that the (unabsorbed) anti-D will mimic an underlying alloantibody.

Lectures

The sixth lecture, "Working with Autoantibodies" (see appendix #4f) is given after the type-and-screen and panel reveals the patient to have a panagglutinin and a positive DAT. The lecture introduces the approach to the problem and solution of an underlying alloantibody. After the case is completed Lecture #7 "Pretransfusion Testing" summarizes the type-and-screen approach to pretransfusion testing.

Specimens and materials specific to the exercise

1. "Patient plasma" containing anti-D.
2. "Patient" RhD-pos RBCs sensitized with anti-D from RhIG in a quantity sufficient for the testing and for elution.
   - Add 0.3 mL RhIG (polyclonal RhIG as marketed in the US is assumed) to 30 mL of R_1R_1 RBCs of the same blood group as the patient plasma and incubate at 37^oC for at least 1 hour.
   - Test DAT and add additional RhIG as needed to increase DAT strength to at least the strength of the antibody screen on the donor anti-D plasma.
3. "Patient" RhD-pos RBCs for adsorption NOT sensitized with RhIG.
   - Variant: "Patient" RhD-neg RBCs for faked adsorption, if an underlying alloantibody is to be mimicked.
4. Faked antibody screening and panel cells consisting entirely of RhD-pos RBCs giving the appearance of a panagglutinin. (If no underlying antibody is to be mimicked, these test cells are also used for the adsorbed plasma).
   - Variant; second "antibody identification panel" prepared from RhD-pos and RhD-neg cells to fake the pattern of an alloantibody labeled to look identical to the "all RhD-pos" panel.
   - Variant; second "antibody identification panel" prepared from all RhD-neg cells to imitate a completely adsorbed autoantibody using saline substituted for ZZAP, labeled to look identical to the "all RhD-pos" panel.
5. Dry transfer bags or 50 mL conical centrifuge tubes for sensitizing RBCs.
6. In-house or commercial ZZAP (or substitute saline, see variant above).
7. In house or commercial eluting reagents.

Procedures

Participants perform each of the following tests. Instructors circulate through the class commenting on students' technique and answering questions. After each test instructors tabulate and analyze the results with the class.

1. Perform a type-and-screen on a patient plasma containing anti-D and all-RhD-pos "faked" set of screening cells.
2. Test the patient plasma against a "faked" all-RhD-pos antibody ID panel including an autocontrol.
3. Perform a DAT.
4. Prepare and test an eluate from the patient's anti-D-sensitized RBCs, including a last-wash test.
5. Perform an autoadsorption including treatment of the adsorbing cells with commercial or in-house ZZAP.
6. Test the adsorbed plasma against a panel (typically the faked all-RhD-pos panel but see variants above and below in "Comments")

Comments

In this exercise the initial antibody screening and panel cells are all Rh positive so that the anti-D-containing plasma appears to be a panagglutinin (antibody reacting with all cells). Similarly when anti-D from the RhIG is eluted from the DAT-positive RBCs it will appear as a panagglutinin with the same fake panel.

A single unit of Rh-positive (R_1R_1) RBCs of the same ABO type as the anti-D-containing patient plasma will suffice for preparing the antibody screening and identification cells, the "patient cells" sensitized with anti-D which are used for the DAT and elution procedure, and for the non-sensitized cells used for the adsorption if the standard option (see "summary" above). A second unit of Rh negative donor RBCs is sufficient for Rh negative adsorbing and panel cells if a variant plan is used.

A common mistake we have made when preparing this exercise is to switch the panel cells to all RhD-positive cells but to neglect to switch the third, RhD negative antibody screening cell.

In the many times we have performed this exercise the uncoated D-positive "patient cells" used for adsorption have successfully removed the anti-D to yield a non-reactive autoadsorbed plasma. However, if there is a concern that the adsorption may not be successful one could test the adsorbed serum against a panel with all D-neg RBCs.

We have only once prepared a panel of antibody identification cells "faked" to yield the appearance of an alloantibody in the autoadsorbed plasma. In this variant the coated "patient" cells must be switched for RhD-neg RBCs so that the anti-D is not removed.

Preparation of both fake panels requires meticulous attention to detail, and the second panel must be labeled to appear the same as the first. In order to preserve the deception required for this case we have typically emptied (and saved) the cells from two commercially-prepared panels used earlier in the workshop and replaced one with the RhD-pos cells needed for the raw (not adsorbed) plasma and eluate and the other with RhD-pos and RhD-neg to mimic the reaction pattern of the "underlying alloantibody". The case stretches over about a day and a half for us, so we can generally prepare the all-RhD-positive panel the night before starting and make the alloantibody-mimicking panel the following night.

Other alternate approaches include:

- Fake the entire adsorption process by switching the anti-D patient sample for plasma that does not contain alloantibody. This can be done any time after performing the initial panel, and typically there is an extended break after preparing and testing the eluate. In this case the ZZAP can be substituted with saline and the all RhD-pos panel can be used to test the "adsorbed plasma".
- Same as above but instead of switching the initial patient plasma (anti-D) for antibody negative plasma, switch it with the antibody used in Case #1 and run it against the real panel used in the previous cases. If this alternative is used, the adsorbing cells must lack the corresponding antigen.

CENTRIFUGE CALIBRATION EXERCISE

Objectives

At the end of the exercise participants will be able to:

1. Articulate the expectations for the performance of a serologic centrifuge, their importance and the principle of centrifuge calibration;
2. Perform functional calibration of a centrifuge used for immunohematologic testing whether it is one designed specifically for this task or a general laboratory centrifuge.

Summary

This exercise has two parts as outlined in the detailed centrifuge calibration procedure included in Appendix 3b. In the first part students perform immediate spin testing with group B donor plasma and reagent group A and B cells using different spin times to achieve an optimal result (clear supernatant, well delineated cell button, cells easily resuspended, etc.) The second part of the exercise evaluates centrifuges performance in the IAT, including both washing and agglutination.

Lectures

None. However, the video demonstrations of reading reaction strengths in tubes (Appendix #7) can be used to discuss the endpoints of calibration.

Specimens and materials specific to the exercise

1. Group A and B reverse grouping cells from the routine "Racks".
2. Group B plasma (anti-A). (Do not use reagent anti-A.)
3. Coombs control cells from the routine "Racks".
4. RhD negative antibody screening cells.
5. 6% albumin

Procedures

Follow the procedures in Appendix #3b.

Comments

In our experience participants are aware of centrifuge speed and time QC but rarely with functional calibration.

EQUIPMENT AND MATERIALS (See also Appendix #2 and #8, below.)

Equipment has been donated or purchased second-hand on the internet as follows.

Serologic centrifuges:
Purpose-built serologic centrifuges which only require 10-20 seconds to pellet RBCs for reading agglutination and 1 minute to pellet for washing are essential. We have used the classic US-built Clay-Adams centrifuges with step-down transformers to convert the 220 current in Indian workshop classrooms to 110. Increasingly these have been supplemented with loaned or purchased Remi Quickfuges™. It is optimal for each participant to have their own centrifuge in order to speed up washing steps, but it is quite acceptable to have 2 participants share a centrifuge.

Dry block incubators at 37^{o}C:
These are readily shared. Capacity must be at least 12 wells per student. If student workstations are on opposite sides of a long table, 4 students can share a single incubator with 48 positions.

We have had problems with 10 mm test tubes purchased in India not fitting heat blocks bored specifically for 10 mm test tubes, so it is best to have blocks bored for at least a millimeter greater than the tubes. For the adsorption case it is also essential to use 13 mm or larger tubes, but only one or two wells are required per student, so one or two aluminum blocks, bored for this size tubes, can be switched out for this exercise and shared by the entire class.

A second problem has been incubators with complicated digital controls. The incubators that have worked best for us are those controlled by a simple analog rheostat. All heat blocks should have a thermometer in a test tube with saline in it. Power should be left on at night so that the incubator temperature is stable.

Although less convenient, water baths could substitute for dry incubators; these would have to accommodate enough test tubes/racks for the entire class.

Thermometers
One thermometer is needed for each 37^{o}C incubator.

Electrical transformers:
The transformer capacity needed is defined by the mix of 110 and 220 current equipment that has been assembled. A large number of power strips are needed to distribute this power to the individual devices.

Test tube and reagent racks
Each student should have their own test tube rack with at least 4 rows of 12 tubes. Reagent racks are shared by pairs (8 racks for 15 recipients, etc.). Both of these items can be purchased from a general lab supply company.

Magnifying mirrors
Simple 10x cosmetic magnifying mirrors can be obtained from some drug or cosmetic stores, or can be purchased online. Each student needs one. Using a magnifying mirror is a good laboratory practice that needs to be promoted.

Timers
Small timers will probably have to be purchased online because of the number needed, one per student. Timers should measure in minutes (e.g. 30 minute incubations) and seconds (e.g. for calibrating centrifuges).

Discard buckets
Two buckets should be available for each student, one for discarded test tubes which will be washed and reused, and one for materials such as pipettes that are discarded. Saline and plasma from reaction washes can be decanted into either bucket. Two buckets for these purposes can be shared by two students sitting next to each other at a table. It is also useful to have a number wastebaskets readily available.

Test tube and pipette containers
Each student needs ready access to a working store of test tubes and disposable plastic pipettes. Clean test tubes can be held in a rectangular plastic food storage container 7 or 8 inches long. These are shared similarly to the reagent racks. A plastic cup or 250 mL saline bottle with its top cut off, taped to the test tube bin will suffice for pipettes.

Saline wash bottles
Each student needs their own. It is convenient to have several wash bottles of a different color if a commercial eluate kit with a special wash solution is used.

Hemostat and scissors
These are needed for transferring specimens from blood component bags and transfer packs.

Supplies and Materials

1. **Pipettes**; the number of pipettes needed can be predicted from the workshop planning spreadsheet.
2. **Test tubes**; the total number needed is predicted by the planning spreadsheet, but typically the tubes used each day are washed at night. If the new test tubes are dusty they must be washed before the first use.
3. **Segment sampling devices**; optional.
4. **Adsorbent paper sheets**; used to cover each workstation. Allow at least 2 per student for replacements.
5. **White tape**; to tape the sides of the adsorbent paper to the worktable(s).
6. **Adsorbent towels**; to clean spills.
7. **Normal saline**; about 24 L for the 5 day workshop, preferably buffered.
8. **Parafilm**; one roll.
9. **Fine point indelible test tube markers.**
10. **Transfer bags and plasma transfer sets**; these are used to facilitate transfer of donor RBCs and plasma to "reagent" bottles and "patient" sample tubes.
11. **Lab coats**; generally provided by the students.
12. **Gloves**

Reagents common to all cases

Reagents such as anti-A and -B are typically supplied in larger volumes than required for each rack, so they are aliquoted into small dropper bottles. The bottles utilized by antibody identification panels for tube testing work well, and the volumes of each reagent needed can be predicted from the planning spreadsheet. Appendix 5 includes labels for the small vials.

1. Anti-A and anti-B
2. Group A and B reverse typing cells (can use RBCs pooled from 3 donors)
3. AHG
4. Coombs control cells
5. ABID RBC panels; If real panels are used, at least two are needed, typically an 11 cell initial panel and a longer panel prepared for confirmation.
6. Enhancement media; as discussed above we have gravitated to use of PEG enhancement.

Specimens

Obtaining the "patient" specimens needed for the workshop is one of the most difficult tasks. The antibody-containing plasmas have come from two sources, blood center reference laboratories which identify them in blood donors, and plasma manufacturers who collect them for manufacture of antigen typing reagents and teaching samples. (The company HemoBioscience in Durham NC markets antibody containing plasma for teaching.) Since blood donors found to have strong plasma antibodies are often unhappy to be deferred or otherwise discouraged from donating, they may be interested in donating in order to support an educational effort. As discussed above we have used plasma containing anti-D from volunteer donors to mimic a warm autoantibody as well as an underlying alloantibody.

This could be done to mimic other specificities. RhIG is a readily available source of anti-D, and although we have only used it to mimic the warm autoantibody eluted in case #5 above, a single vial of RhIG could probably be used to mimic the cases other than the ABO discrepancy if appropriate dilution studies were done. The ABO discrepancy could be faked with plasma and RBCs of the appropriate ABO type switched to mimic the desired reactions in the ABO type and antibody screen.

We carry antibody-containing plasmas in our luggage in shampoo or other such small bottles with tightly sealing tops appropriately packaged in Ziploc bags with adsorbent material.

Compared to antibody-containing plasmas, "patient RBCs" are relatively easy to obtain. For RBCs of specific phenotypes extra segments can be made, with additional tubing added using a sterile connecting device if needed. RBCs needed in larger volume such as the RhD-positive and RhD-negative cells in case #5 units of RBCs are generally provided by the host blood bank.

Workbook

The student workbook contains a title page, the workshop schedule and objectives, all of the worksheets on which the students will record their work on the exercises including antigen profile for the screening cells and panels, a QC worksheet and a centrifuge calibration worksheet, a short procedure manual including a chart depicting different agglutination grades, and pages for notes. The workbook is spiral bound with a clear plastic cover and heavy back. Exercises and other sections are separated by colored paper.

Each case starts with a patient coversheet adapted from one of our laboratories', in part as a suggestion to our participants to use *some sort* of a coversheet to organize the multiple pages of panel sheets and other worksheets used by their own laboratories, including selected cell panel sheets, phenotyping sheets, titrations sheets, etc. The case coversheet also includes blank matrix that can be designated for recording reactions with screening cells or other selected cells performed by multiple techniques, and written explanations what cells, sera, and methods were employed.

Appendix 3a consists of a single WORD document of the workbook pages in the order in which they appear in the III workshops; this file can be divided up into separate files for the individual pages and printed or duplicated. Appendix 3b is a WORD file of the student procedure manual which can be customized for your own workshop and printed for each workbook. Two sided printing/duplication will cut down on the weight of the workbooks, which is important if a large number must be carried on an airplane.

Other workshops

It may be difficult to obtain sufficient quantities of the antibody specimens listed above for all participants to work with each of them. Dr. Ankit Mathur (Rotary ***ttk*** Blood Bank, Bangalore) came up with a clever solution using multiple smaller volumes of antibody specimens with differing specificities handed out to the participants. They ran their antibody screens by the gel method and analyzed their results as a group. They then performed a first panel by the same panel by the same method and again analyzed the results. In some cases the identification could be completed after antigen typing, but some required selected cells from a second panel the results of which were again shared with the group. This gave all of the participants a similar hands-on experience, but in one exercise allowed them to analyze multiple cases in a much shorter time, probably in a single day. This could even be combined with specimens showing an ABO discrepancy. If additional days were available, the group could then go on to an autoantibody case, as in case #5 above, for a relatively complete introduction in as few as 2½ days.

We have conducted two workshops focusing just on autoantibodies. The most recent was 3 days in length to investigate one cold- and one warm-reactive autoantibody. The cold autoantibody workup included prewarmed testing, a thermal amplitude test, and a cold agglutinin titer. The warm autoantibody workup included warm autoadsorption and was identical to case #5 above.

GENERAL NOTES:

Don't overwhelm the participants with extraneous detail!

Be prepared for slow progress in the first day or day and a half. The participants will be becoming familiar with the laboratory setup, class protocol, result documentation, as well as performing manual tube tests. With each exercise the process will become more streamlined for most participants.

There will frequently be several participants who will quickly grasp the skills and concepts. More advanced discussions may be held with these individuals during their down time; however, it should be emphasized that they should not work ahead in the exercises. You may be able to use such individuals to help those having difficulty understanding, particularly if the problem is a language barrier. Nonetheless you must monitor their learning to make sure the concepts being conveyed are correct.

Similarly, there may be technically proficient students, usually experienced technologists/laboratory scientists, who race off into a problem before you have thoroughly explained it. It is important to notice when students are working when you are making an important announcement and to explain that we will work no faster as a class than the slowest person.

It's hard to have too many small dropper vials of the size provided with antibody identification cell panels as discussed above

Although it is tempting to have a copy or printing shop duplicate, assemble, and bind workbooks, it has been our experience that if one has a good copier, given that some pages will be copied/printed double-sided and some single sided, it may be better just to assemble/collate the workbooks yourself, and then only have the copy shop do the spiral binding. In addition to making a workbook for each participant, make workbooks for each faculty member and any observers.

Although our initial intention was to use saline/tube testing since it is the most economical for the participants' laboratories', and because it includes reading after the 37^{o}C incubation which increases the observation of antibody behavior, nonetheless we have gravitated towards using PEG enhancement for the alloantibody cases because the

enhanced agglutination reaction strengths are easier to read for less experienced participants and because it saves incubation time.

Our suggestions for "faking" or mimicking the samples were generated from difficult experience with obtaining antibodies of the appropriate specificity in the appropriate quantity. They are not, however, the only way samples may be mimicked. You can be creative in other ways to accomplish the same goal. It will be every important that you carefully plan each deception that is intended so that the instructors themselves don't get confused. Even if the use of real antibody-containing plasma is planned, it is wise to have extra anti-D plasma or reagent available. This way a sample can quickly be faked if the original plasma loses reactivity.

Another challenge comes when 120V US-wired equipment is being used in a 220V environment. It is extremely important to label EVERY cord with the name of the equipment and its voltage. The path of each cord must be followed carefully to insure that it is not plugged into the wrong type of power. Plug strips must be labeled with the voltage they are plugged in to. Participants must be instructed NEVER to alter the plugs once set up. As our experience generally has been with a mix of 120V and 220V equipment, the importance of this planning cannot be overemphasized. It will always take longer to set up the electrical connections than is expected. It is good to have 1-2 more transformers and several more plug adaptors than are expected to be needed.

List of Appendices:

Appendix 1: Workshop Schedule, WORD document
Appendix 2: Workshop materials planning spreadsheet, EXCEL document.
Appendix 3a: Workbook worksheets in WORD format; these are provided as a single WORD file which can be divided up into individual single page files, one for each worksheet.
3b: Procedure manual to be included in the workshop workbooks, with NOTES pages at the end.
Appendix 4a: Lecture #1, "Hemagglutination testing; Grading Reactions"
4b: Lecture #2, "The Indirect Antiglobulin Test"
4c: Lecture #3, "Blood Group Antibody Identification
4d: Lecture #4, "Rule out Cell Exceptions"
4e: Lecture #5, "ABO discrepancies"
4f: Lecture #6, "Working With Autoantibodies"
4g: Lecture #7, "Pretransfusion Testing"
Appendix 5: Sheets for printing labels for reagent dropper bottles.
Appendix 6: Evaluation form, example
Appendix 7: Short video demonstrations of reaction grading
Appendix 8: Wet workshop equipment list example

Typical Workstation