Test Procedures

C/255 7C21

Not all, but most Immunogenetic work, has been done with particulate antigens, specifically erythrocytes (red blood cells = RBC). Two main tests are involved: (1) the agglutination test, (2) the hemolytic test.

1. Agglutination tests:

   The antigen is held constant and the antibody titered -- the reverse of precipitin tests. Another difference: multiple injections are usual in the recipient.

General standard procedure (does vary):

1. Fill out protocol or test sheet headings.
2. Wash RBC in isotonic (0.75 - 0.92%) saline 3 times in 10 volumes (or more).
3. Resuspend to 3% (later by eye.)
4. Titer antiserum in doubling, quadrupling or tens dilutions.
5. Add two drops (pipette drops 20-40 drops - I prefer 30 per ml) to the tubes or reaction wells, horizontally: 1st row undiluted, 2nd row 1/2, 3rd row 1/4, etc. for doubling dilutions. The last row should be a saline control.
6. Wash pipette by aspiration between dilutions (and later between cell samples).
7. Add one drop of the 3% washed cell samples to each dilution vertically straight back (i.e., perpendicular to the horizontal rows of antisera.)
8. Shake well.
9. Read at 5 minutes, 30 minutes, and 2 hours after the setup. Agitate or shake well after each reading. Check for cold agglutinins by leaving overnight in the refrigerator, 4°C. By precendent the 5 minute reading is recorded in pencil at the top of the square, the 30 minute reading is taken in permanent blue or black (India ink preferred) in the lower left, the 2 hour reading is taken in red lower right) and the overnight reading (if any) in green.

Cold agglutinins are rare. They are characterized by reacting only at low temperatures, 4°C, and not at room temp. 25 ± 2°C or at 37°C, body temperature. Further, the reaction disappears if the test is warmed to room temperature. Further, they are absorbed out only at the low temp.

Agglutination is read by gentle shaking or swirling the mixture, and distinguishing grades of clumping of the red cells. The degree of agglutination is relative and slightly subjective.

. = 0 = all cells disperse freely - no clumping (need microscopic verification).
± = a few very small clumps — " " " (may have some free cells noticeable)
+ = many definite but small clumps — (may have some free cells noticeable)
++ = many definite clumps — (may have some free cells noticeable)
1 = many larger clumps — no free cells
2 = several large clumps — " " "
3 = a few large clumps — " " "
4 = one large clump — " " " (2-3 large clumps permissable)

Example: anti-sera against RBC of pigeon titered against 2-3% washed RBC.

dilution:	U	1/2	1/4	1/8	16	32	64	128	256	512	1024	2048	4096	8192	16,384
typical reaction:	0	2	3	3	4	4	4	4	4	4	4	3	++	±	.

(0 2 3 3 = prozone)

Dots are preferred to represent zero's since the reactions then stand out obviously on the test sheet.

2. Hemolytic tests. Generally the same procedure is used as in agglutination, except after the antigen-antibody is mixed, one drop of complement is added and the test shaken. Hemolytic tests are best performed in tubes. Readings are taken at 30 minutes, at 2 hours, and at 4 hours after setup. The latter readings require the cells to have settled completely to the bottom. This takes about 1 1/2 hours fot cattle erythrocytes. Earlier readings are sometimes pertinent. Two controls are used: saline control, complement control, and sometimes normal sera selected for reactivity with "bacterially activated" cells.

2 hours

Hemolytic readings include two aspects simultaneously: the quantity of red cells left and the redness of the supernate. Further, two series of recording are used: one for the early 1/2 hour reading and another for fully settled reactions.

For nonsettled mixtures:

. = 0 = all cells intact, clear white supernate.
± = some lysis present (cloudy red); but some red cells still intact.
4 = all cells lysed; solution clear red.

For fully settled mixtures:

. = 0 = all cells intact, clear white supernatant.
± = no easily detectable loss of red cell volume, but tinge of red present.
tr = no easily detectable loss of red cell volume, but definite trace of red.
1 = about 25% red cells lysed, and the rest intact; supernatant red.
2 = about 50% red cells lysed, and the rest intact; supernatant quite red.
3 = about 75%-99% red cells lysed; supernatant very red.
4 = all cells lysed; solution clear cherry red (occasionally some reagents leave "white"-appearing cell envelopes visible weakly agglutinated.)

Test Procedures: Obtaining blood samples for Immunogenetic tests.

Generally, there is no intention of deliberately killing the animal by any of these means. Even heart puncture in rabbits, for example, is supposed to result in death only 5% of the time. Small mammals such as guinea pigs, hamsters and mice are more easily bled after anaesthesia. However, rabbits and chickens reputedly have greater losses if anaesthesia is used.

Many methods are known and used. Demonstrations save prolonged descriptions! The following are among those preferred:

1. Intracardially-used on all species smaller than turkeys - preferred when large quantities are needed. Strap down or restraining boards are used if the operator works alone or if the assistants are not skilled in holding the animals. For rabbits and chickens an 18 gauge l~" needle (Huber point preferred) is used on a 50 ml syringe; and fifty ml of blood can be removed from adult rabbits and chickens normal sized or larger.

2. From the jugular vein - used on cattle, sheep, horses, goats, hogs and Gallinaceous poultry (not ducks nor pigeons). Large quantities (3-5 liters) are obtained from cattle by use of vacuum bottles and double needle rubber h~es.

3. From the arm (brachial or wing vein) - used -in humans and birds. Needle and syringe may be used; or in birds only, the vein may be sliced across near the elbow and the blood allowed to drop into a tube.

4. From the ear - used on rabbits only, although it should work on hogs and cattle for small amounts using needle and syringe. In rabbits xylol is put on the ear if much is wanted, then the marginal ear vein is sliced about 1/2" along its length. The flow toward the heart is stopped by thumb and finger pressure and the blood dropped into tubes.

5. By toe or tail clip - used on small birds or rodents. Good for small amounts only.

Test Procedures: Processing Antisera from Whole Blood.

During an immunization series, periodic (e.g., weekly) serum samples should be tested, since then one can determine when the titer is appropriately high. In practice this is not always feasible. Immunization duration is then based on past successes. Most generally, three to four weeks of injections are appropriate for immunizations against red cells.

At the end of the immunization series of injections, a waiting period of about one week (usual range 4-10 days after the last injection) is allowed, hopefully for the antibody titer to rise to its maximum. The recipients then are bled in quantity.

Usually the blood is collected in clean dry glass bottles and allowed to clot. In mammals the clot shrinks expressing serum in a few hours' time. Placing the fresh clots at 37°C for the first two hours is helpful in getting the best yield. The clots should be broken away from the side of the tube at about half an hour if they have not already done so naturally. For large clots (300 ml or more), longer times are necessary for full clotting and shrinking. The serum is poured off at about 3-4 hours, centrifuged clear, often recentrifuged clear, then labeled and stored frozen at -20°C or colder. If the clots are allowed to stand overnight at room or refrigerator temperatures, additional yields may be obtained. The later yields are not demonstrably different in antibody content but are more contaminated with bacterial growth and should be heated at 56°C for 30 minutes. They are then conveniently complement deactivated and, therefore, ready for testing and absorbing.

Blood clots from birds are quite variable in shrinking and expressing serum. When saline agglutinating tests only are involved, it is more rewarding, generally, to obtain plasma from birds. The dilution effect is small and the yield considerably increased. Fibrin in the plasma will precipitate out after each freezing and thawing, sometimes obscuring reactions. The fibrin may be removed by heating at 56°C for 30 minutes and <u>then</u> standing 48 hours at refrigerator temperatures and finally centrifuging the fibrin precipitate out.

Always <u>label</u> completely antisera to be stored more than two or three days.

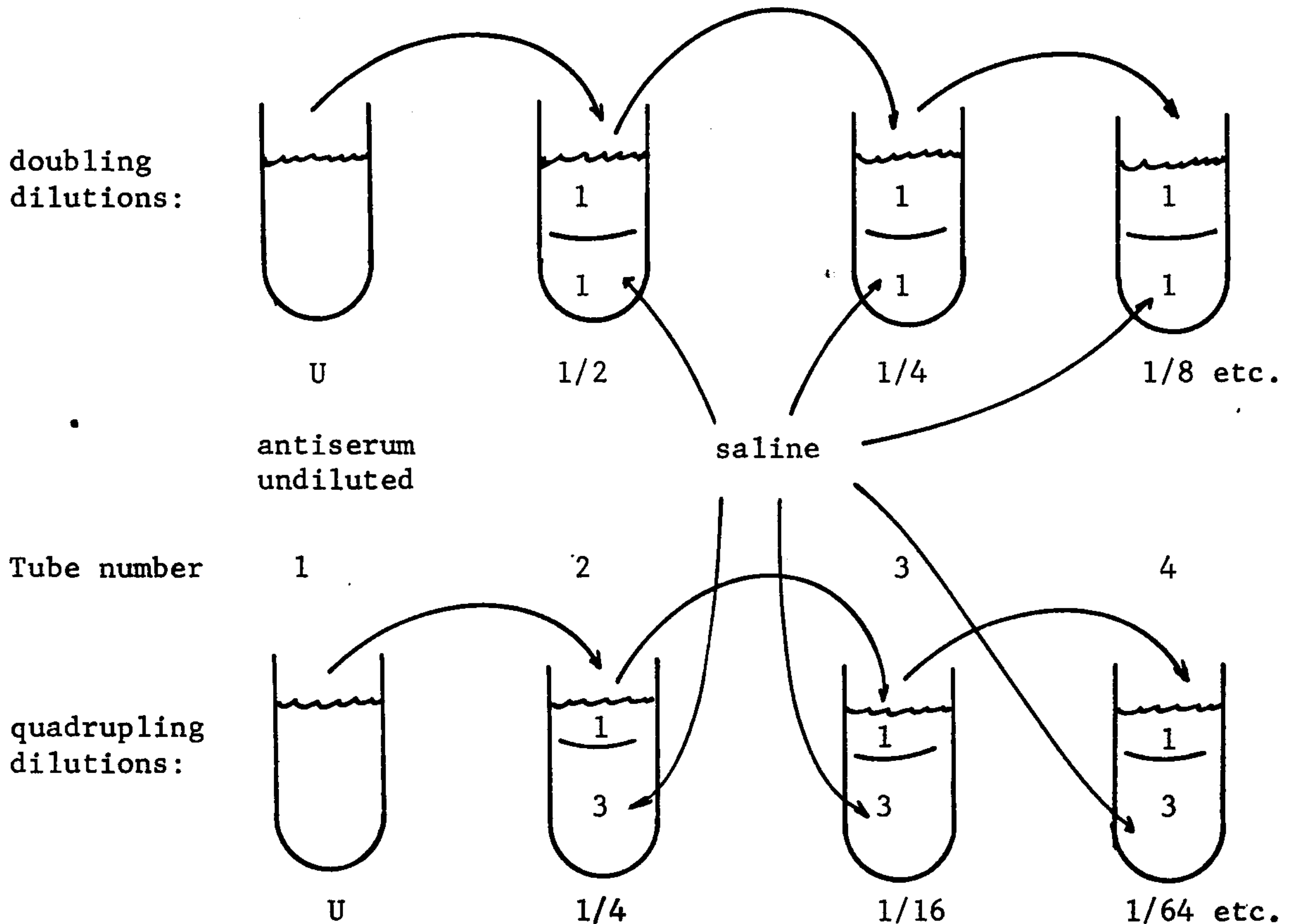
Test Procedure: Titers

The titer of an antiserum is the highest dilution which still (agglutinates) reacts with the (homologous) test antigen.

It is often expressed as the reciprocal or the denominator of the actual dilution e.g. $\frac{1}{512} \doteq 512$, or as the number of doubling or quadrupling from undiluted. U = 1   1/4 = 2   1/16 = 3. . . . which conserves space and is more quickly meaningful.

Replicate tests indicate that a difference of one doubling dilution **is not usually significant.**

Method:



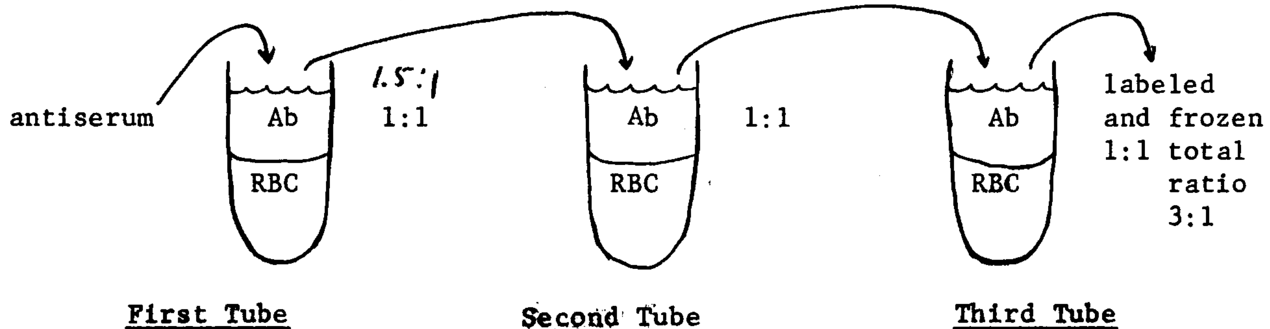
Test Procedures: Absorption

Absorptions are used to fractionate the serological specificities, that is, to reduce the number of specificities present in sera. Absorptions are also used to demonstrate or prove presence or absence of an antigenic factor (identical or cross-reactive) not disclosed by more ordinary reactions.

Absorptions are often characterized by the absorbing ratio of the total volume of washed packed red cells to the volume of antisera at a selected dilution. For example, an absorbing ratio of 3:1 means a total of 3 volumes of packed cells to 1 volume of antisera. However, absorptions are always performed in 2 or more tubes whenever a ratio higher than 1:1 is used. The volume of packed cells should never exceed the volume of antiserum in any one tube. Two to 4 volumes of antiserum to packed cells in one tube is commonly used.

Successive absorptions of an antiserum are performed until the cells no longer react with the reagent (except for particular subytpe fractionations). In absorptions of saline agglutinating antibodies with red cells, the first absorption tube usually exhibits rapid and strong agglutination, easily seen on the sides of the tube. Such absorption series should nearly always be carried out two tubes beyond the visible reaction. The amount of absorptions of "blocking" type antibodies is usually based on previous experience. In either case, a direct test is necessary to tell whether or not the absorption is complete.

A typical absorption of rabbit anti-dove at 1/16 dilution heated 56°C 1/2 hour (unabsorbed titer 1/4096):



First Tube

Antibodies mixed with packed red cells of pigeon for 5 minutes, then centrifuged, cells discarded.

Second Tube

Supernatant from 1st tube mixed with red cells for 30 minutes, then centrifuged, cells discarded.

Third Tube

Supernatant from 2nd tube mixed with red cells for 1 hour, then for 1/2 hour at refrigarator temperature, then centrifuged, cells discarded, and test fluid removed and tested or stored frozen.

To avoid homolysis, centrifuge the absorption mixture as soon as agglutination becomes obvious. This is especially important for the hemolytic tests.

Δ unless desorption is made

Test Procedures: Inhibition test

An antiserum of strong titer (example: anti-J of cattle, 1/512) is selected and a dilution (e.g. 1/8) made in saline for which positively reacting cells should exhibit total reaction.

A typical strongly reactive cell type is also chosen, washed and suspended in saline to 3%. Another cell type or more are similarily prepared for replication, and/or if the cell varistion is important.

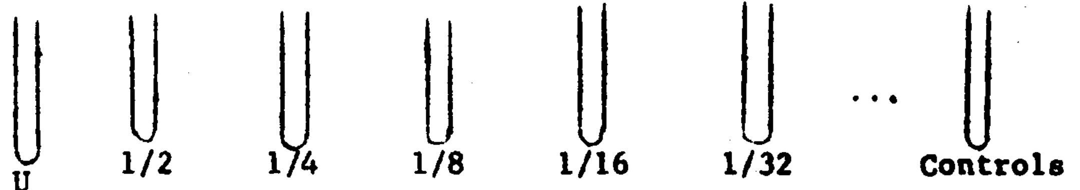
The inhibitant (e.g. serum from suspected J+ cattle) is diluted in a series of doubling dilutions with saline.

Two drops of the antiserum at 1/8 are placed in each tube except the saline control. Then one drop of inhibitant is added and mixed with the antiserum in titer of the inhibitant dilutions. After "incubation" (standing at room temperature) for about 15 minutes, one drop of red cells is then added and mixed (shaken) and incubated for about 5 minutes. Then selected (rabbit) complement (undiluted) is added and the mixture shaken and read at the appropriate times.

If the inhibitant contained a specificity reactive with the antibodies, it will "block" those antibodies from reacting with the known reactive cells, until the dilution is too great to inhibit the red cell reaction.

Resumé:

all tubes (except inhibitant control) 2 drops of antiserum at 1/8



One drop of inhibitant added in titer of dilutions

one drop of red cells

One drop of the complement is added to all tubes except the saline control, which gets four drops of saline. The complement control gets three drops of saline. The antibody control gets one drop of saline. Thus, saline replaces either antibody or complement or the inhibitant or all, so that all tubes have five drops volume.

Some principles and major conclusions of classical immunogenetics:

1. Antigens exhibit species and individual differences (polymorphism).

2. Antigens are genetically controlled. The preponderant number of antigens are dominant to their absence or codominant to each other. But both recessive antigens and epistasis occur.

3. The genetic alternative for an antigen is usually another antigen (antithetical relationship).

4. Several genetic systems may be involved in controlling the phenotypic expression of one antigen.

5. A variety of immunological specificities may be elicited by one simple haptenic group.

6. The symbolism developed for antigenic factors cannot be related to the true character of the immunizing antigen. That is, the serological complexity does not imply the genetic complexity.

7. Species-specific and individual differences may be controlled by the same genetic system.

8. Species-specific antigens are seldom identical in two or more species; but related specificities (heterophil antigens) are often widely distributed in animals and will elicit cross-reacting antibodies.

9. The pattern of reaction of different reagents among different samples often implies the genetic control.

10. Tests with antigens in solution, such as serum, reflect very well only the larger taxonomic groupings such as families and suborders.

11. Antibody titers for particulate, cellular, antigens are ~ additive.

12. Interactions of alleles ~ non-alleles may yield new specificities, i.e. interaction products.

13. Cross-reactions may be non-reciprocal!

14. Many immunogenetic specificities occur in particular groups, phenogroups, which are inherited as a unit via the controlling genes; exceptions being of mutational- like frequencies.

15. The "same" (cross absorbable) antigenic factor in different phenogroups may exhibit quite different and characteristic strengths of reaction, titer and antigenicity in immunizations.

16. Tissue antigenic differences are responsible for tissue graft rejections.

17. Developmental (ontological) differences occur for some antigens and not for others. 18. Immunogenetic

systems may be medically important, and may be used for parentage analysis.

19. In some diploid microorganisms the expression of one antigenic type may be switched to another (phase variation) by environmental changes.

20. In vertebrates heterozygous for allotypic phenogr~ups only one allele in one immunocytic cell line may be expressed (allelic exclusion).

21. There may be several reasons why membrane antigens exist, but none yet have been generalized unequivocally.