

ANSC150 - Laboratory # 6

IMMUNOLOGIC METHODS

Lab will start with a brief quiz based on the content of the handout.

There is some preparatory homework required that will take about an hour to complete. Read the handout and complete the portions so-indicated before your scheduled lab.

There will be some additional work to complete from your homework within the lab and you may wish to add notes to the handout during the discussion. The handout is to be passed in for grading the week after your lab section.

The lab will be organized around the following:

1. Lab Quiz
2. General Description of Immune Methods
3. Response to Immunization (take-home)
4. Radioimmunoassay (take-home)
5. Diagnostic Immunoassay Tests (perform test in lab)
6. Questions and Discussion for Each Section.

RESPONSE TO IMMUNIZATION

This exercise illustrates one method for quantifying the response of an animal to immunization. The overall method will be outlined and a data set estimating "antibody response" is provided for your analysis. We speak of antibody levels in terms of "titers", or the extent to which an antiserum can be diluted, while still exhibiting the capacity for interacting and binding with antigen. The logic is straightforward -- if the endpoint is the ability to complex a given amount of antigen, a very rich source of antibody can be much further diluted (or extended), compared to a less concentrated source of antibody. This method can be visualized in the "diffusion through agar" format described in **Figure 1** where any detectable line of reaction between the wells is the endpoint. Consider the following: the central well contains a convenient concentration of **Ag** and the peripheral wells (1-4) each contain increasing dilutions of the test antiserum (decreasing amounts of **Ab**). A precipitin line will be most evident for the most concentrated **Ab**, less obvious for the next dilution, and then finally will be undetectable at some dilution. We can crudely estimate titer of this antiserum on the basis of the greatest dilution still resulting in some detectable precipitation.

As will be discussed in class, immunosaturation assays permit precise quantification of reactions between **Ab** and **Ag**. This method, using radioactively-labeled **Ag** ($^{125}\text{I-Ag}$) has been employed in our example described in the next two pages. Dilutions of test serum were incubated with a fixed amount of $^{125}\text{I-Ag}$ (amount expressed in radioactivity units, e.g., counts per minutes, or c.p.m., determined by an instrument that "counts" radioisotope emissions). Once equilibrium binding has been reached, radioactivity in the **Ab**· $^{125}\text{I-Ag}$ complex is measured after separation from non-reacted **Ab** and non-reacted $^{125}\text{I-Ag}$. For convenience, these values are usually converted to percentages (fixed amount originally added equals 100%) and % binding in the **Ab**·**Ag** complexes can range between 100% and 0%. We will define "titer" as our best estimate of the dilution of antiserum providing for 50% binding of the $^{125}\text{I-Ag}$. The % binding usually relates in a linear fashion to the \log_{10} of the dilution of antiserum, so if values for % binding (ordinate - Y-axis) are plotted against dilution (abscissa - X-axis) on log graph paper, a straight line can be fitted by eye and the dilution expected to bind 50% of the $^{125}\text{I-Ag}$ can be read off the abscissa. Since this is a log scale and some judgement is required when reading the scale, a "dummy" data set is plotted in the attached example figure. Be certain that you understand how to use the log scale when plotting and how this graph has been used before proceeding. Set the log paper horizontally in front of you such that the log scale grid is from left to right and the linear grid is vertical. Once the points are plotted and the "best-fit" line drawn, interpolate the titre (1:2840 in this example) by reading the dilution corresponding to 50% binding.

Your exercise: You will examine the time-course of antibody response to a typical immunization schedule used to prepare useful immune serum from an experimental animal. Let's imagine that a researcher has successfully isolated and purified a novel protein from the placenta of sheep. The protein has been called ovine Placental Protein (oPP for short). The researcher has sufficient oPP to raise antibodies in a rabbit and the plan is to use these antibodies for future research. A very small quantity of oPP is mixed with **adjuvant**, a mixture of emulsified chemicals that enhance the immune response and this mixture is aseptically injected under the skin of the rabbit. Sufficient oPP is saved to permit re-immunization (called **boosting**) 5 weeks later. The researcher is optimistic of obtaining a very large response in terms of antibody production.

In our example, the researcher may be excited about the progress and wants to monitor the dynamics of the antibody response. On weeks 1, 2, 3, 4, 5, 6, 8, 11 and 14, a small blood sample is collected from the rabbit, allowed to clot and the sera are stored away for later analysis. From experience, the researcher guesses that maximal antibody titers will be obtained about 14 days after the booster immunization (i.e., on week 7) so a sizeable blood sample is collected at this time.

After all the sera samples are collected and are ready for testing, a very tiny quantity of oPP is used to make ^{125}I -oPP to be used as radioactive **Ag** in titer-testing. From each of the thawed sera, 10 microliters are diluted to 1.0 ml (1:100 dilution), then further diluted to 1:330, 1:1000, 1:3300, 1:10000, 1:33000 and being really optimistic!) to 1:100000. So, with sera from 10 bleeds, each with 7 dilutions, our researcher incubates a fixed quantity of ^{125}I -oPP with a constant volume of each of the 70 unknowns. After a couple of days, the **Ab**• ^{125}I -oPP complexes are separated from non-bound labeled oPP and the radioactivity is counted. The % binding of the ^{125}I -oPP is calculated and the tabulated data is listed in the table below. The columns indicate sera, identified by week of bleeding, while the rows correspond to dilutions of each serum.

TITER TESTS Rabbit 407 Immunized with oPP										
	Week of Bleed									
	1	2	3	4	5	6	7	8	11	14
Test dilution	Percent binding of ^{125}I -oPP									
1:100	91	100	98	98	95	100	100	100	100	100
1:330	64	74	76	72	68	100	100	100	100	100
1:1000	41	50	49	47	44	91	100	100	99	98
1:3300	14	24	25	21	18	65	76	77	74	76
1:10000	0	1	2	0	0	41	52	53	50	51
1:33000	0	0	0	0	0	15	26	27	24	25
1:100000	0	0	0	0	0	0	3	4	0	2
Titer										

DO THE FOLLOWING BEFORE COMING TO LAB:

1. Use the 3-cycle log paper to plot % binding against dilution, as in the example figure, but each student will plot just 3 columns to avoid excessive clutter. Do not use an excessive number of points along each axis.

If your last name begins with A, E, I, M, Q or U, plot the data for weeks 1, 5 and 11.

If your last name begins with B, F, J, N, R or V, plot the data for weeks 2, 6 and 14.

If your last name begins with C, G, K, O, S or W, plot the data for weeks 1, 3 and 7.

If your last name begins with D, H, L, P, T, X, Y or Z, plot the data for weeks 4, 8 and 11.

2. Interpolate each of the lines (draw the 3 lines and read off the abscissa) to estimate the dilution providing 50% binding for each week that you have plotted. This is your estimate of titer and the values should be listed in the appropriate spaces along the bottom of the table. During the lab, the TAs will collate the various estimates of titer that have been derived by all the students so that you can complete steps 3-5 in this assignment.

Continue with other work to be done before lab by going on to Radioimmunoassay.

Items 3-5 below will be done in class during your lab section.

3. Using the regular linear graph paper, construct a simple plot that illustrates the change in antibody titer against weeks after the first immunization. Use common sense to select which variable is to be on the ordinate (dependent), and choose a scale that makes best use of the paper and the divisions in the fine grid. Draw a line that connects the points, starting with a titer of 0 at the time of first injection.

4. "Read" the graph and be prepared to be called upon to briefly describe what the data reveal. Such a description would show the timing of peaks and their duration in weeks.

5. Now, think about the meaning of the data so that you can contribute to a discussion of the following points:

Why is the plot started at 0,0?

How long would the response to first injection have lasted if there had been no boosting?

Was the response to boosting predictable? Why?

RADIOIMMUNOASSAY

The radioimmunoassay (**RIA**) has been the most widely used immunosaturation method in animal science, diagnostic endocrinology, drug testing and forensic sciences. It is being overtaken by Enzyme Linked Immuno Saturation Assays (**ELISAs**) and other enzyme immunoassays (**EIA**). All these procedures take advantage of the binding of **Ag** by specific **Ab** and will be described in class. The RIA remains more reliable because of the precision with which radioisotopes can be measured. As described in lecture, any immunosaturation assay relies upon the finite binding capacity of a suitable dilution of antiserum for its antigen. Highly selective antibodies are used, and in RIAs, we need a radioactive form of the antigen (**Ag***) and some reliable means of distinguishing antibody-bound **Ag*** (**Ab•Ag***) from non-bound **Ag***. **Sensitivity** is the smallest quantity of **Ag** that can be reliably distinguished from no **Ag**. In practice, we usually accept 90-93% binding as different from no **Ag** or 100% in an RIA standard curve plot.

The assay depends upon competition between a fixed quantity of labeled antigen (**Ag***) and a variable quantity of unlabeled **Ag** for binding to a fixed number of binding sites in the **Ab**. Known quantities of **Ag** are used to construct a **calibration**, or **standard curve** with unknown (or test) samples being interpolated off this curve. When equilibrium binding between **Ab** and **Ag** (mixtures comprised of a fixed amount of **Ag*** and variable amounts of **Ag**) has occurred, the amount of **Ag*** bound by **Ab** is determined. The standard curve takes the form of the amount of **Ag*** being linearly and inversely related to the log of unlabeled **Ag** in the tubes making up the standards.

Samples suspected to contain amounts of the **Ag** are substituted for reference quantities of **Ag** (but otherwise handled exactly the same way). The amount of **Ag*** bound for these "unknowns" is then simply interpolated from the standard curve and read off in **Ag**-concentration equivalents.

The data set provided here is taken from a RIA for progesterone, an important steroid hormone derived in large part from the corpus luteum on the ovary. Animals were immunized to progesterone to develop a high AB titre capable of binding radioactive progesterone (**Ag***). (Similar to the Response to Immunization Exercise.) A standard curve was prepared using known concentrations of pure progesterone that ranged from 0 to 10 ng/ml. This unit (ng/ml) means nanograms (10^{-9} grams) per milliliter. The tabulation of data includes the amount (counts) of radioactive progesterone bound for each of 8 levels of the standard (i.e., unlabeled **Ag** including 0 progesterone). The level of radioactivity for each of 11 unknown plasma samples, assayed exactly as were the standards, is also provided. Blood samples were obtained every couple of days from a cow during the estrous cycle. The interval between the days on which estrus is observed is 21 days -- the day of estrus is labeled 0 or 21. If you

are unsure about the meaning of *estrus* and *estrous cycle*, refer to your text book.

RADIOIMMUNOASSAY DATA

<u>STANDARD CURVE</u>		<u>PLASMA SAMPLES FROM A COW</u>	
Concentration (ng/ml)	Counts*	Sampling Day of cycle	Counts* Concentration (ng/ml)
0	11900	0	10900 ...
.02	12000	1	9900 ...
.06	11800	3	8200 ...
.20	9400	5	4600 ...
.60	6500	7	3300 ...
2.0	4100	9	2900 ...
5.0	2000	11	3100 ...
10.0	400	13	3100 ...
		15	3000 ...
		17	2900 ...
		19	7300 ...
		21	10700 ...

*Radioactivity (**Ag***) bound to **Ab**

TRY TO COMPLETE THE FOLLOWING BEFORE LAB:

1. Plot the progesterone standard curve on 3-cycle log paper with 10 ng/ml on the far right hand side of the log scale (total range of scale is .01 - 10 ng/ml). Plot counts on the Y-axis using a linear scale that makes maximal use of the size of the paper. Be sure to label both axes. Draw a straight line that accurately fits as many points as possible (it won't fit them precisely and some points may be way off). Near the top and bottom you can curve the line to fit more points (Best-fit" line).

2. Since the 0 value cannot be plotted on a log scale (log of 0 does not exist), why was the 0 value in the data set?

3. What is the **sensitivity** of this assay? Sensitivity is defined within this handout and let's use 90% of the 0 level for this answer. Sensitivity = _____ ng/ml.

4. Use the standard curve to interpolate the progesterone concentrations in the cow plasma samples and write them along side the data set in the table above.

5. Plot plasma concentrations of progesterone against Day of the Estrous Cycle on regular graph paper. Hint: Before starting, carefully consider what any graph attempts to depict. Examine other graphs (e.g., in the scientific papers you are reading, or in the text book) to help you decide the best way to decide on the choice of axes, scaling and sequence of days.

6. Interpret the data (graph) and answer the following questions in the space provided here.

a. High levels of progesterone are measured during the phase of the estrous cycle when the corpus luteum is active. Which range of days are these?

b. What is special about the day on which levels were the lowest? Can you think of a practical use for the observation that you have just made?

c. Why might another cow display consistently high levels of progesterone, starting from day 9 and continuing for at least another 30 days when we stopped collecting samples?

For bonus points: What is another reason related to a reproductive disorder?

DIAGNOSTIC IMMUNOASSAY TESTS

The availability of specific antibodies to various hormones and many other molecules has led to the development and availability of many new diagnostic assays. Some of these are so simple that they can be used at home, e.g., drug store early pregnancy tests (EPT). Many of these procedures do not require radioisotopes and this can be an important advantage.

The new immunoassay-based tests can be exquisitely sensitive like a radioimmunoassay or only qualitative (yes or no). The choice here depends on whether the result desired will be used for research, more testing, or clinical treatment *versus* having knowledge of whether the factor being tested for is present or absent. Perhaps you can think of examples for each preference.

Examples of various immunoassay test procedures include: Enzyme Linked Immuno Saturation Assays (ELISA), Enzyme-immunoassay (EIA), immunoagglutination tests and immunoblotting techniques. The latter is an important technique for molecular biology, but will not be discussed here. The test procedures utilize enzymes linked to an antibody to yield a product detected by color changes in a spectrophotometer. The big advantage here is that the radioactive tracer used in radioimmunoassay is replaced by an enzyme. Everything else about these assay procedures is similar to RIA. The enzyme

activity is monitored by adding substrates that result in color changes and this reaction is quantified. The information coming from a standard curve can be used in just the same way that a radioactive tracer was used earlier in this laboratory exercise.

One of the simplest and easiest immunoassay procedures used today is the ELISA. You will perform this test in lab to quantitatively assess the content of progesterone in cow's milk. The test will determine whether the milk samples contain high or low amounts of progesterone and thus provide information about the reproductive function of the cow.

a. Read how the Accufirm test works in the excerpt from the test brochure attached.

The test results are based on the competition between the amount of progesterone in milk (high levels or below some lower level). We could use this test to sequentially monitor progesterone production over days of the estrous cycle just like was done in Part 3. This same type of assay is widely used for pregnancy tests in women using urine.

b. Look over the instruction sheet for the Accufirm test. The TA's will instruct each of you in performing the test. Your data will be summarized along with that from your lab section. Since samples will be positive or negative, observe the tests to be sure you recognize both reactions.

c. Milk samples were collected from 3 cows labeled as day 1 and day 21. These cows are labeled A, B and C with B-1 and B-21 being the samples collected from B cow on days 1 and 21, respectively. You will be given additional information in lab about breeding dates.

d. Answer the following questions during lab:

1) Which cow is likely to be pregnant?

2) Which cow was inseminated at an inappropriate time and therefore is unlikely to be pregnant? What further information would be required to make a final judgement?

3) Which cow cannot be pregnant? Why?

4) Think about the collective results in your lab section and be prepared to discuss the value of such testing to a dairyman.

Since each test costs \$4, do you think the benefits might be cost effective?