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The Use of Radioimmunoassay for The Study of Australia Antigen

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Our laboratory has developed a two-step, direct radioimmunoassay for detecting and studying Australia antigen. We wish to report the nature of the test and some of the results that we have found upon analyzing blood specimens from hepatitis patients and normal blood donors.

The two-step, dirct radioimmune assay is adapablte to antigens with multiple determinents. Therefore, let us first consider the nature of the Australia antigen. It is found in human blood and is associated with an agent that will produce the clinical disease of serum hepatitis when an antigen positive blood is infused into a recipient. The antigen was discovered in the early 1960's by Dr. Barry Blumberg of Philadelphia. In his studies, at that time, he was seeking antigens which would react with antibodies formed in the blood of mutliply transfused hemophiliac patients. He found that multiply transfused patients developed antibodies against serum beta lipoproteins. In further studies on the variations of lipoproteins, Blumberg discovered an antigen in the serum of an Australia aborigine that was not a lipoprotein, but still reacted strongly with the serum of a multiply transfused hemophiliac. He called this new antigen Australia antigen.

Later the Australia antigen was found to be rather widespread. It was found in apparently normal persons, and was also observed in patienrs with several diseases. However, the most consistent association of Australia antigen was with patients with acute viral hepatitis.

Patients receiving units of blood containing Australia antigen have a very good chance of developing serum hepatitis. Thus, there has been a great demand to screen blood and blood products for Australia antigen to prevent their infusion into a recipient.

Some of the properties of Australia antigen are shown on the first slide.

AUSTRALIA ANTIGEN, PROPERTIES

- PARTICLE, 25 nm
- DENSITY, 1.20 gm/cm^3
- ETHER SENSITIVE
- HEAT STABLE, 56°c/1 hr.
- NUCLEIC ACID, ?

Fig. 1.

Australia antigen in the electron micrscope is a particle appearing uniformly of about 25 nanometers in diameter. It has a buoyant density of 1.20 grams per cubic centimeter; it is seinsitive to ether; it is heat srabile at for one hour; it looks very much like a virus; as of now there has been no consistent demonstration that highly purified preparation of Australia antigen contain nucleic acid. Neither RNA nor DNA has as yet been found.

In slide 2 we will see an electron micrograph of a highly purified preparation of Australia antigen showing that indeed it looks like a small virus. Most of the particles are small spheres but one always finds a number of tubules of varying length.

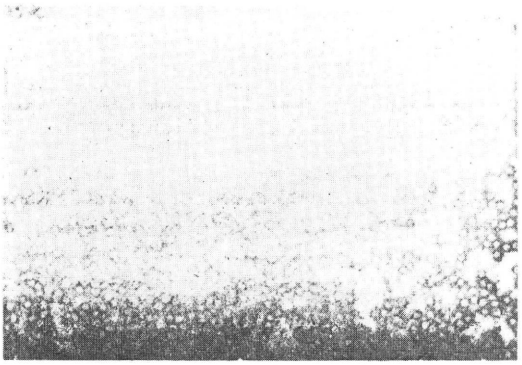


Fig. 2.

Since the agent cannot be grown in tissue culture, and there are as yet no animal models exhibiting infectivity, one must use some form of immunologcal or biophysical test. The next slide shows a listing of the tests that have been applied to Australia antigen.

AUSTRALIA ANTIGEN DETECTION

- IMMUNODIFFUSION (AGD)
- ELECTRON MICROSCOPY (EM)
- COMPLEMENT FIXATION (CF)
- HEMAGGLUTINATION (HA)
- IMMUNOELECTROPHORESIS (CIEP)
- RADIOIMMUNE ASSAY (RIA)

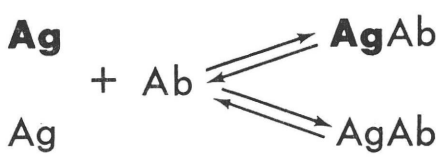
Fig. 3.

Immunodiffusion (AGD) is a conventional agar gel diffusion in which a precipitin line is formed between antigen and antibody. Secondly, the antigen can be identified in the electron microscope; many blood samples have tiers high enough to be observed directly, but usually some form of concentration must first be used before electron microscopy. One of the most sensitive of the immunological methods is complement fixation, and this has been applied to Australia antigen. The Australia antigen itself dose not hemagglutinate red cells; however, a passive hemagglutination can be developed by sensitizing red cells with the antigen. These sensitized cells will agglutinate with antiboby. Antigen can be detected by inhibition of hemagglutination. Immunoelectrophoresis (CIEP) has been successfully applied to Australia antigen and is about 5 times as sensitive as procedures based on immunodiffusion. Finally, the most recent application of a testing procedure for Australia antigen it radioimmune assay. This was first reported by Dr. Yalow in 1970. and has proved to be the most sensitive of all methods.

Our particular use of the radioimmune assay for Australia antigen will be the subjecet of the remainder of this talk. Let us first review essential features of the conventional radioimmune assay. As shown on the next slide, this is generally based on isotope dilution. Normally, an antigen in a purified form is labeled with a radioisotope of iodine, either iodine ^{113}I or iodine ^{123}I, signfied on this slide as bold-face AG. When the radioactive antigen reacts with a lmited amount of antiboby, an antigen antibody complex is formed. The amount of an unknown can be estimated by competition for a limited amount of antibody. Thus, as shown here, when an unknown amount of an unlabeled antigen is present, along with a labeled antigen, two complexes are formed, one radioactive and one not. The amount of the radioactive complex is in direct proportion to the relative amount of labeled and unlabeled antigen in the original sample.

The actual measurements are mede after separatioa of the labeled antigen-antibody complex from un-

RADIOIMMUNE ASSAY
Isotope Dilution (Indirect)



SEPARATION

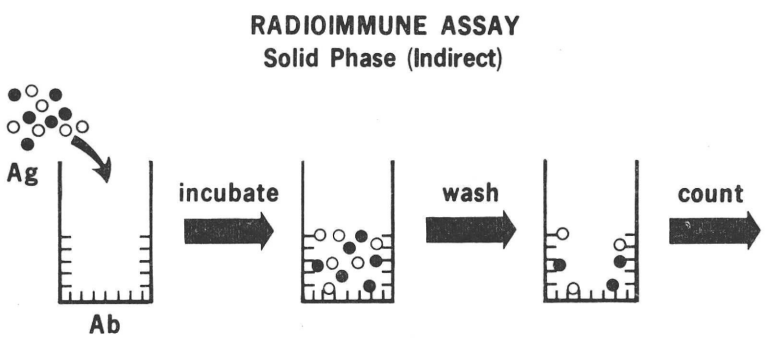
- ELECTROPHORESIS
- DOUBLE ANTIBODY PRECIPITATION
- SOLID-PHASE ANTIBODY

Fig. 4.

reacted labeled antigen. Electrophoresis is frequently used. The antigen normally will move, having a net positive or negative charge. and the antigen antibody complex will not move or will move very slowlv. Another method of separation of antigen antibody complex from unreacted antigen is based on using a double antibody precipitation. In this case, an antibody to the gamma globulin used in the reaction is added and this then will precipitate the antigen-antibody comlex while not affecting the free antigen. Separation then is based on centrifugation of the precipitate to remove it from unrected antigen. Finally, a third way of separating antigen-antibody complexes from unreacted antigen is based on solid phase antibody. This was first developed by Dr. Catt in which he found that antibody could be bound to a solid phase; and when it reacted with antigen the complex would be found in the solid phase and could be separated from unreacted antigen by simply washiag away the soluble antigen.

In its simplest form, the solid phase antibody is bound to the surface of a plastic tube. Reaction with antigen takes place at the surface. After incubation the unreacted antigen is simply removed by washing. The tube then contains the antigen-antibody complex fixed to the surface.

This general procedure is shown in diagramatic form on the next slide. This is radioimmune assay,



Fig, 5.

solid phase, indirect; indirect meaning that again there is competition for a limited amount of antibody by labeled and unlabeled aetigen. Here, there is a tube in which antibody has been fixed to the surface. In the first step, a mixture of labeled and unlabeled antigen is added to the tube. And, after a suitable incubation time, as we see in the second step, a number of both labeled and unlabeled antigens will be fixed to the antibody bound to the surface of the tube. The relative amount of labeled unlabeled antigen bound antigenbound to the tube will, of course, depend upon the original ratio of labeled and unlabeled antigen. After a suitable incubation time, the unreacted materials are washed from the tube and the tube is counted. The amount of unlabeled antigen in the original samble can be estimated by the decrease in counts from a similar sample in which there was no unlabeled antigen in the sample. Note in this example that we started with an equal mixture of labeled and unlabeled antigens, six of each. In the final tube, three labeled and three unlabeled antigens were fixed.

Using a variation of the solid phase antibody technique we have developed a two-step procedure for radioimmune assay as illustrated on the next slide. In this case, one starts again with a tube in which specific antibody has been fixed to the surface. In step 1, a known amount of antigen or a sample containing an unhnown amount of antigen is added to the tube. After a suitable incubation period a number of the antigens will be fixed to the tube in an antigen-antibody complex. The unreacted antigens are washed from the tube, as shown in the last example of the top line. In step 2 of this procedure we can ask: were there antigens fixed to the tube? If so, how many? For this procedure, one adds to the

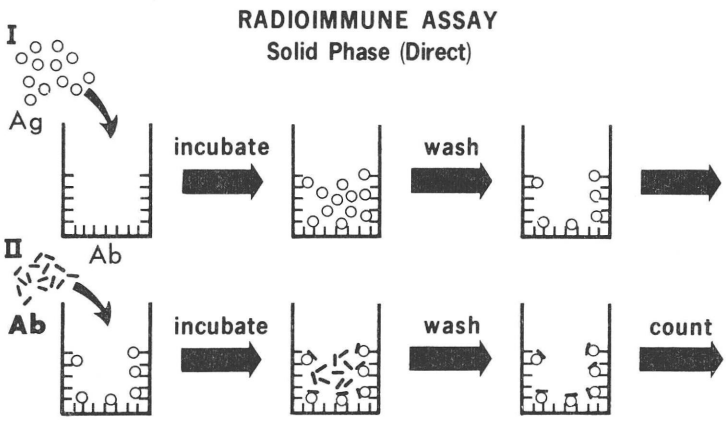


Fig. 6.

tube a large excess of specific antibody, labeled with iodine ^{125}I. After a suitable incubation period a number of iodine-labeled antibody molecules will react with available determinants on the viruses fixed to the surface of the tube. After this incubation period, the tube is again washed, removing unreacte labeled antibodies; and the tube is finally counted. Here, one can easily see that the amount of antigen fixed to the tube in step one is in direct proportion to the number of iodine-labeled antibody molecules that are fixed to the tube in the second step.

There are several advantages of the solid phase direct radioimmune assay over those based on competition of unlabeled antigen with labeled antigen in a single reaction. First, in the solid phase, direct method, a high concentration of antigen can be used in step one. This improves the kinetics of the antigen, antibody interaction. In the case of Australia antigen. undiluted blood serum is used in volumes up to 1 ml. Secondly, in step two a high concentration and a large excess of iodine ^{125}I-labeled antibody may be used. This large excess favors an incrased number of counts being fixed. The additional counts improve the sensitivity of detection. A third advantage of the two step procedure is that antigen can be detected even in the presence of antibody. In the case of viruses, the simultaneons presence of both antigen and antibody is a frequent occurrence in blood.

Let us now look at some results, comparing the direct radioimmune assay with other methods of detecting Australia antigen. For these studies, we made a preparation of highly purified Australia antigen. This highly purified material was then diluted in two-fold increments into normal human serum. Each dilution was then tested for the presence Australia antigen by five different detection procedures. The results are shown in the next slide. In the first column on your left the concentration of Australia antigen is recorded. This varies from 80 μg/ml in the most conceutrated sample. to 0.005 μg/ml in the least concentlated sample In the second colum, the results with the direct RIA test that we have been discussing are recorded in counts/minute. The counts varied from 12,000 in the most concentrated samples, down to 496 for the 5 μg/ml sample. The lowest positive result was 833 counts in the sample that contained 10 picograms of Australia antigen per ml. The third column gives the results using the antibody-coated tubes in the indirect procedure. In this case, the larger concentration of Australia antigen, the lower the counts. Following this column downward, one can see that the last sample in which a positive result was found, was a level of 0.15 μg/ml. In the foruth column, the lowest level detected as positive by complement fixation was 2.5 μg/ml. Next, the counterimmunoeletrophoresis procedure was less sensitive, detecting only 5 μg/ml. And, finally, the agar gel diffusion technique showed a positive result at

RELATIVE SENSITIVITY

Au Conc. µg/ml	RIA (DIRECT) CPM	RIA (INDIRECT) CPM	CF 1/DIL.	CIEP + OR −	AGD + OR −
80	10961	261	64	+	+
40	12208	370	32	+	+
20	10482	401	32	+	+
10	10447	558	16	+	+
5	11253	656	4	+	−
2.5	11291	1193	2	−	−
1.25	11169	2640	−	−	−
0.6	10765	3455	−	−	−
0.3	10080	5640	−	−	−
0.15	8082	5672	−	−	−
0.075	4536	8833	−	−	−
0.04	3198	7761	−	−	−
0.02	1302	6493	−	−	−
0.01	833	9737	−	−	−
0.005	496	7927	−	−	−

Fig. 7.

10µg/ml. The radioimmne assay by the direct method was thus 1,000 times more sensitive than AGD, 500 times more sensitive than CIEP, 250 times more senstive than CE, and about 16 times more sensitve than the solid phase indirect technique.

In order to demonstrate that the solid phase procedure could be used to detect antigen even in the presence of antibody, we took a standard amount of purified antigen and added increasing amounts of anti-Australia antigen serum to it. Each sample was then tested for antigen and antibody by the radioimmune procedure. The results of these tests are shown on the last slide. In colum one is the ratio of antigen to antibody. This varied from, 0, in which no antibody was added, to a level of 128 equivalents of antibody. Column two gives the results of the antigen test. The level of antigen in the sample containing no antibody was recorded as 8,390 counts per minrte. As increasing amounts of antibody were added, such as 0.5 equivalents, the sample was still positive for antigen. At one equivalent of antibody, the counts were 9,712, again showing a positive result. With two eqnivalents of antibody, the counts were still positive. At four equivalents of antibody, the counts were 2,236, still giving positive results. However, when eight equivalents of antibody were added, the counts were not above background, and the antigen was no longer detectable. Although I have not discussed it thus far, it is possible to test for antibody by procedures very similar to what I have described for antigen. In this case, antigen is fixed to the tube, and ^{125}I-labeled antigen is used in the second step. In the third column of this slide, the results using such a procedure to test for antibody are recorded. The counts when no antibody were added, which would be background counts, were, 350. At a half equivalent of antibody there was no increse in counts, and this amount of anti-

ANTIGEN-ANTIBODY MIXTURES

	Ab/Ag RATIO	Ag TEST (CPM)	Ab TEST (CPM)	
	0	8390	350	
	0.5	7892	324	−
	1	9712	1107	+
	2	7357	2724	
+	4	2236	3546	
−	8	484	6325	
	16	362	6053	
	32	425	12650	
	64	405	11144	
	128	468	12740	

Fig. 8.

body was not detectable. However, beginning at one equivalent of antibody all levels were detectable as illustrated here by increasing number of counts. Thus, it is seen that in mixtures of antigen and 1 equivalent, 2 equivalents, and 4 equivalents of antibody, both antigen and antibody in the same sample, can each be detected and quantified using the solid phase direct method that I have described.

I have described results of our use of a radioimmune assay in a very specialized case, the Australia antigen. However, we believe that the technique described would be equally applicable for radioimmune assay for any antigen that has more than one available combining sight for antibody. This would include, certainly, almost all of the viruses that we know about. The procedure, so far, in our hands is more sensitive than any other test.

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