The Production of Antibodies to Australia Antigen in Mouse Ascites Fluid[1] (34705)

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Australia antigen, Au(1), a particulate antigen associated with acute and chronic hepatitis (1), was first reported by Blumberg in 1964 (2). There is growing evidence that Au(1) is, or is an antigen on, a form of hepatitis virus (1, 3–6). The antigen reacts with human and rabbit precipitating antibodies of the gamma G type. The antisera which were originally described have been designated anti-Au(1) (7, 8).

Levene and Blumberg (9) in 1968 reported an additional type of Australia antiserum designated "anti-Au(2)." The anti-Au(2) antiserum which was produced in hyperimmunized rabbits reacted with a greater percentage of sera from hepatitis patients than did anti-Au(1). Our suspicion that this antibody might be of the gamma M (19S) type came about as a result of three observations. First, the titer of anti-Au(2) decreased with time in the frozen state, even though the initial titer was high. Second, the anti-Au(2) disappeared after rabbits that had produced anti-Au(2) were reinjected with Australia antigen. Third, anti-Au(2) could never be demonstrated in the gamma G fraction eluted from DEAE Sephadex with 0.02 *M* phosphate buffer at pH 8.0. It is known that IgM antibodies can detect antigenic determinants not detectable by IgG antibodies (10).

Munoz (11), in 1957, reported that the intraperitoneal injection of mice with antigen incorporated with complete Freund's adjuvant resulted in the production of large volumes of ascitic fluid which contained antibodies specific for the injected antigen. Lieberman (12) in 1959 confirmed Munoz's results substituting Staphylococci for the Mycobacteria in the Freund's adjuvant. In 1962, Berkovitch (13) reported that the Munoz technique could be used to produce viral antibodies. With the latter studies in mind, we undertook a study to determine whether mice could produce precipitating antibody to Australia antigen and also whether antibodies of the gamma G and gamma M types could be demonstrated.

There are three results of the investigations reported here. First, the production of antibody to Australia antigen in mouse ascitic fluid; second, the demonstration that these antibodies are both gamma M and gamma G; and third, the demonstration that the specificities are similar but not identical to the antibodies of anti-Au(1) of human origin.

Materials and Methods. Mice. The mice were pathogen-free females of the Charles River strain, weighing 10 to 12 g.

Antigen. Pooled sera of 2 patients was used as antigen. One patient was an 18-year-old male with Down's syndrome, anicteric hepatitis and Australia antigen in his serum. The other was a 37-year-old female with chronic renal disease, who had received multiple transfusions while undergoing hemodialysis and who developed anicteric hepatitis with Au(1) in her serum.

Immunization. One hundred mice were injected intraperitoneally with 0.1 ml of a mixture containing 1 part pooled Australia antigen sera and 3 parts complete Freund's adjuvant (Baltimore Biological Laboratories). Six weeks later, all mice were given a second intraperitoneal injection of 0.1 ml of a mixture containing 1 part pooled Australia antigen serum and 1 part complete Freund's ad-

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juvant. Forty percent[2] of the mice died within 24 hr of the second injection. Two to 4 weeks after the second injection, more than 50% of the surviving mice developed ascites. Large amounts of ascitic fluid were removed by injecting air into the intraperitoneal cavity with an 18-gauge needle and allowing the fluid to drain into a beaker through the needle. Up to 10 ml/mouse could be removed at each tap. Mice were tapped several times and the rate of ascitic fluid production could be increased by periodic intraperitoneal injection of 0.1 ml of a 1:1 mixture of saline and complete Freund's adjuvant.

Immunodiffusion tests. The immunodiffusion tests were performed with the use of the micro-Ouchterlony technique described by Blumberg and Riddell (14) and the micro-micro technique of Krause and Raunio (15).

Immunoelectrophoresis. Agar immunoelectrophoresis was performed on 3.25 × 4.25-in. lantern slide plates layered with 1.1% agarose prepared with Veronal buffer, pH 8.4, ionic strength 0.03 (16). Mouse ascitic fluids or sera containing Australia antigen were electrophoretically resolved in 40 min (80 V, 25 mA). The resolved proteins were then allowed to react by immunodiffusion for 24 hr at room temperature either with human sera containing Australia antigen or with goat antimouse serum (Behringwerke Diagnostic Reagents, Woodbury, N.Y.).

Column chromatography. Gamma globulin was separated from mouse ascitic fluid as described by Sela *et al.* (17). In addition, ascitic fluid proteins were resolved by gel filtration through Sephadex G 200 in a 60 × 2.5-cm column (Pharmacia, Uppsala, Sweden). Elution was carried out with 0.85% NaCl.

Detection of antibody. Australia antigen specific antibodies in ascitic fluids were detected by two methods both involving the absorption of ascitic fluid with normal human serum to produce a reagent reactive only with Australia antigen. In the first, ("in-well absorption"), normal human sera (not containing Australia antigen) were first placed in wells and allowed to diffuse into the agar for 30 min. Then the mouse ascitic fluid containing antibody to Australia antigen was placed in the same wells. The sera to be tested for the presence of Australia antigen were placed in the peripheral wells. The second method was that of bulk absorption. The least amount of normal human serum necessary to absorb all antibodies except those reacting with Australia antigen was first determined. On the basis of this preliminary titration, a larger volume of mouse ascitic fluid was absorbed with the calculated volume of normal human serum. This is similar to the method of Melartin and Blumberg (8). Absorbed ascitic fluids were tested against a panel of sera scored as positive and negative for Australia antigen using a standard human and rabbit anti-Au (1).

[2] We have since learned that mortality could be considerably decreased by changing the first injection mixture from 3 parts to 2 parts of Freund's adjuvant.

Mercaptoethanol treatment. Absorbed mouse ascitic fluid was added to an equal amount of 0.01 *M* mercaptoethanol in 0.02 *M* phosphate buffer, pH 7.4. The mixture was incubated for 1 hr at 37° and then at room temperature overnight. As a control, equal amounts of mouse ascitic fluid and physiological saline were mixed and incubated for 1 hr at 37° and then at room temperature overnight.

Results. Absorbed mouse ascitic fluids show lines of identity with human and rabbit antisera to Australia antigen (Fig. 1). In order to characterize the specificities further, we compared the reactions of mouse and human anti-Australia antigen with 2813 sera. This follows the general method of Melartin and Blumberg (8) and will be elucidated in greater detail in a future publication from this laboratory. The results are shown in a 2 × 2 contingency table (Table I). The mouse ascitic fluid antibodies reacted with 84 of 111 (76%) specimens that reacted with human antiserum. Six of the sera reacted with mouse ascitic fluid antibody, but did not react with human antiserum. The high chi-square value (χ^2 = 1935.) indicates that there is a strong association between the Australia antigen identified by use of the antibodies in mouse ascitic fluid and that identified by human antisera. The results also show that

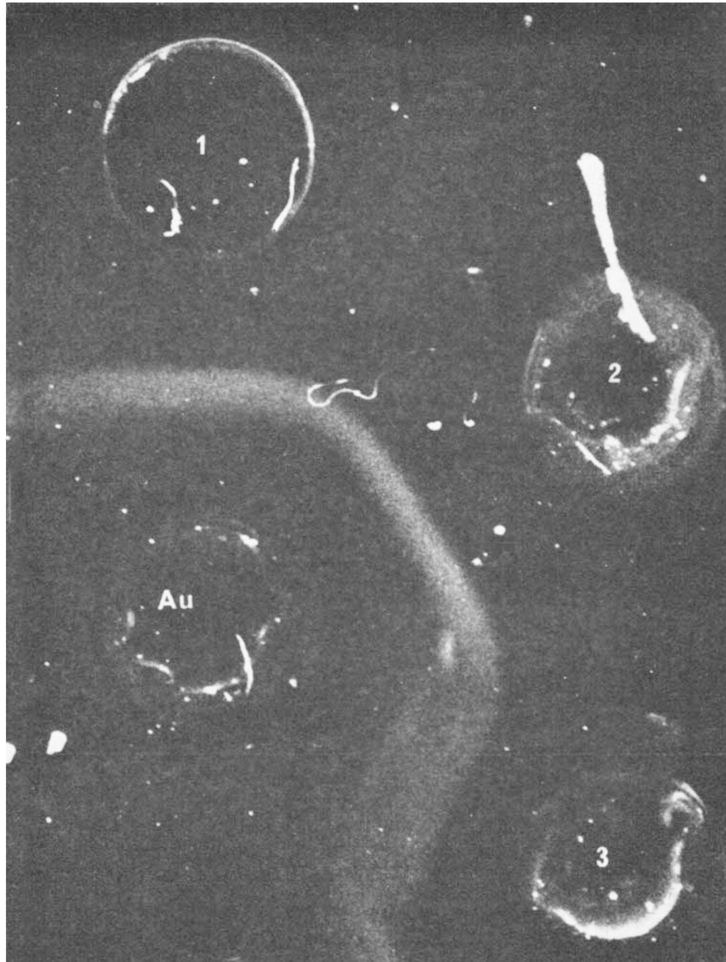

FIG. 1. An Ouchterlony immunodiffusion plate showing the line of identity between mouse, human and rabbit antibodies to Australia antigen. The well designated Au contains reference Australia antigen. Well No. 1 contains human antisera to Australia antigen; well No. 2 contains mouse ascitic fluid containing antibodies to Australia antigen; and well No. 3 contains rabbit antisera to Australia antigen.

TABLE I. Comparison of Mouse Ascitic Fluid Antibodies and Human Antibodies to Australia Antigen.[a]

	Mouse antibody	
	+	—
Human antibody		
+	84[b]	27
—	6	2696

[a] $\chi^2 = 1935$; $p << .001$.
[b] Represents 76% of the Australia antigen positive sera.

there are multiple antibodies in mouse ascitic fluid and human antisera. Further, while these antibodies identify determinants that are similar, they may also identify determinants that are not.

We next attempted to ascertain whether mouse ascites antibodies were of the gamma G or gamma M type. Three ml of pooled mouse ascitic fluid were placed on a DEAE Sephadex column and the gamma G fraction was eluted with 0.02 *M* phosphate buffer, pH 8.0. The gamma G fraction was then concentrated to the original volume by lyophiliza-

tion, and compared with the unfractionated mouse ascitic fluid pool by means of immunodiffusion titration. Unfractionated mouse ascitic fluid pool produced a precipitin band in dilutions up to 1:6 while activity in the gamma G fraction could not be demonstrated in dilutions above 1:2. This decrease in activity could be explained either by retention of some gamma G on Sephadex or by the retention of anti-Australia antigen gamma M. Evidence for the latter was shown by the following experiment. Three ml of mouse ascitic fluid pool was subjected to gel filtration through a Sephadex G 200 column. Those fractions comprising the first peak contained anti-Australia antigen as demonstrated by immunodiffusion.

Ascitic fluids from individual mice were next tested to determine if differences in specificity could be detected. Some mice, tapped several times, produced as much as 100 ml of ascitic fluid. Several of the ascitic fluids produced double precipitin bands when reacted with certain sera containing Australia antigen. The latter produced only a single precipitin band when reacted with human, rabbit, and our first pool of mouse ascitic fluids.

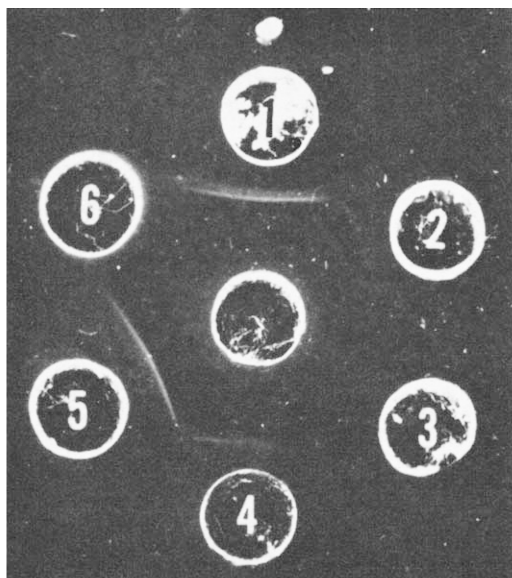

FIG. 2. An Ouchterlony immunodiffusion pattern demonstrating the various types of reactions with the mouse ascitic fluid designated AF 6 in the center well and sera containing Australia antigen (positive when tested with human antisera to Australia antigen in the peripheral wells. For further explanation, see text.

In Fig. 2 one of these ascitic fluids (AF 6) is placed in the center well, and sera containing Australia antigen which reacted with human anti-Au (1) are in the peripheral wells. There is a double band between the center well and wells 1 and 5; these wells contain the serum of a Down's syndrome patient (M.C.). There is also a precipitin reaction between the center well and wells 2 and 4 which contain the serum from a second Down's syndrome patient (J.B.). However AF 6 does not react with the sera in well 3 or in well 6, each of which contains serum from a third patient who has Australia antigen in his serum. To determine whether one of the two precipitin bands was due to another Australia antigen determinant or to a normal component of M.C.'s serum (a result of incomplete absorption) the following experiments were conducted:

(i) M.C.'s serum was subjected to immunoelectrophoresis. AF 6 was next placed in the adjoining trough to allow development of the protein bands by immunodiffusion. A double precipitin band in the region of the alpha 2 globulins was found; this is the region where Australia antigen migrates (18).

(ii) Specific antisera to alpha 2 macroglobulin, ceruloplasmin, haptoglobin and Gc globulin (Behringwerke Diagnostic Reagents, Woodbury, N. Y.), all serum proteins which migrate to the alpha 2 globulin position, produced precipitin bands of non-identity with those of M.C. and AF 6.

(iii) AF 6 absorbed with a serum containing Australia antigen no longer produced precipitin bands between AF 6 and serum M.C. AF 6 absorbed with normal human serum (not containing Australia antigen) did not eliminate the two precipitin bands. These findings strongly support the possibility that both precipitin bands were formed by antibodies to antigenic determinants of Australia antigen.

Further evidence that one of the two precipitin bands produced by reacting AF 6 with M.C. was gamma M (gamma M was shown to exist with the pooled mouse sera de-

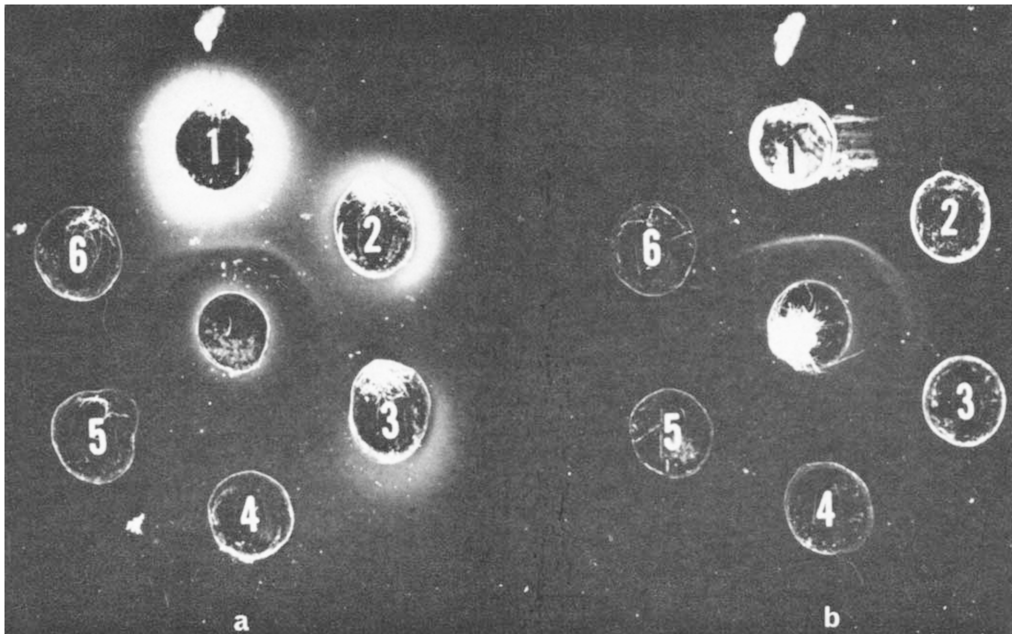

FIG. 3. A comparison of mouse ascitic fluid, AF 6, treated with mercaptoethanol with saline as a control. The center wells contain Australia antigen. The peripheral wells of the Pattern A (wells 1–6) contain mercaptoethanol treated ascitic fluids (AF 6) in dilutions of 1:1, 1:2, 1:3, 1:4, 1:5, and 1:6, respectively. The peripheral wells in the Pattern B contain the same dilutions of saline treated ascitic fluid (AF 6).

scribed above), was the results of treatment with mercaptoethanol. While mercaptoethanol lability does not provide proof that a globulin is gamma M, it lends support.

Figure 3 (A and B) shows results by immunodiffusion of treating AF 6 with mercaptoethanol. M.C.'s serum (containing Australia antigen) was placed in the center wells and dilutions of the treated AF 6 were placed in the peripheral wells. The mercaptoethanol treated AF 6 (Fig. 3a) and the control (Fig. 3b) produced precipitin bands in dilutions of 1:2. However, only one precipitin band was produced by the mercaptoethanol-treated AF 6, while two bands were produced by the control (Fig. 3b). This is consistent with the explanation that at least one of the two bands is gamma M globulin.

Discussion. Mice immunized with Australia antigen in complete Freund's adjuvant by the intraperitoneal route produce large quantities (100 ml/mouse) of antibody which react with Australia antigen by immunodiffusion assay. These antibodies are similar to anti-Au(1) produced in humans and rabbits (8), although they are not identical in specificity. This method of producing antibodies provides another source of anti-Au(1) in addition to rabbits and humans. More than one determinant of Australia antigen was detected with antibodies contained in mouse ascitic fluid. Double precipitin bands, one due to gamma M and another due to gamma G represent two separate specificities.

The major portion of antibody reacting with Australia antigen as demonstrated by immunodiffusion assay in mouse ascitic fluid is gamma M. This was demonstrated by DEAE Sephadex retention and the fact that the major activity (immunodiffusion) is found in fractions of the first peak after gel filtration through Sephadex G 200. Furthermore, it was shown that one of two reacting Australia antigen–antibody precipitin bands was eliminated by prior treatment of the ascitic fluid with mercaptoethanol, which is known to destroy gamma M antibodies.

From this study it appears that gamma M,

presumably 19S, is involved in reaction with multiple determinants of Australia antigen. Cowan (10) has shown that 19S antibodies differentiate immunologically different strains of foot and mouth disease virus. Our data do not rule out the possibility that the gamma M moiety of mouse ascitic fluid is 19S gamma G as found in the piglet and described by Kim *et al.* (19). The possibility is currently under investigation that the anti-Au(2), originally described by Levene and Blumberg (9), was of the gamma M type and similar to some of our mouse ascitic fluids.

Summary. Antibodies to the Australia antigen were shown to be present in the ascitic fluid of mice hyperimmunized with Australia antigen–Freund's adjuvant mixtures. As much as 100 ml can be removed from individual mice if they are restimulated by intraperitoneal injections of adjuvant–saline mixtures. The antibodies contained in these ascitic fluids were shown to be both of the gamma G and gamma M types. The specificities of the antibodies contained in the mouse ascitic fluid were shown to be similar but not identical to human antibody to Australia antigen.

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