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DETECTION OF AUSTRALIA ANTIGEN BY BIOLOGICAL ASSAY IN 'Au NEGATIVE' KIDNEY HOMOGRAFT RECIPIENTS WITH HEPATIC DYSFUNCTION

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SUMMARY

Rabbits were immunized with the sera from patients with known Au antigenaemia; from kidney homograft recipients who were Au negative with direct conventional tests but who had hepatic dysfunction; and from normal volunteers. Heterologous anti-Au antibodies were raised by the first of these kinds of sera but not by the third. Sera from seven of the eleven immunosuppressed kidney recipients of the second group did not raise anti-Au antibodies in the rabbit, but sera from the other four did. The results indicate that the Au antigen may be present in trace quantities in significant numbers of immunosuppressed patients previously thought to be Au negative. However, they do not unequivocally establish an aetiologic association between Au antigenaemia and 'post-transplantation liver disease', probably because of the obscuring and important factor of hepatotoxicity of the immunosuppressive agents.

INTRODUCTION

Post-transplantation liver disease is a well-recognized complication of renal homotransplantation under immunosuppression. A clear association between this kind of liver disease and the presence or absence of the Australia (Au) antigen could not be established in a recent study from our institution (Torisu *et al.*, 1971). Particularly troubling was a group of eleven recipients who had severe or even fatal hepatic damage but who never had the Au antigen in any of multiple stored postoperative serum samples. The present study using a heterologous antiserum system was designed to see if some of these eleven patients did, in fact, have the Au antigen which was missed because of the insensitivity of the detection methods.

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METHODS

Detection of Au antigen

The Au antigen and anti-Au antibody were looked for in all subjects entered into this study with Ouchterlony's (1958) micro-method (AG), immuno-electro-osmophoresis (IEOP) (Bussard, 1959; Prince & Burke, 1970), complement fixation (CF) (Sever, 1962; Shulman & Barker, 1969) and haemagglutination (HA) or haemagglutination inhibition (HAI) (Vyas & Shulman, 1970) tests. In addition, anticomplementary activity (ACA), which is thought to measure circulating Au antigen anti-Au antibody complexes, was determined (Mayer, 1961; Shulman & Barker, 1969; Torisu *et al.*, 1971).

Case material

Au antigen and anti-Au antibody standard sources. Two renal transplant recipients under immunosuppression with chronic Au antigenaemia provided sera with high titre reference antigen. Human anti-Au antiserum was donated by a third kidney recipient who had developed chronic aggressive hepatitis in the post-transplantation period without ever having demonstrable Au antigen.

Control patients with Au antigenaemia. Multiple serum samples were collected from five additional patients with the Au antigen. Three of these patients were under immunosuppression after renal homotransplantation, but did not have severe hepatitis. The other two, who had never received immunosuppressive therapy, had typical acute serum hepatitis.

Patients without Au antigenaemia. Sera from eleven kidney recipients with post-transplantation hepatic dysfunction were collected starting about 1 week before the onset of liver dysfunction and continuing until 1 week after recovery or, in three instances, until death. These cases, which have been previously reported (Torisu *et al.*, 1971), are listed in Table 1.

Negative controls. The sera from 200 healthy volunteers were pooled. These individuals were all Au-negative by the complement fixation test.

Preparation of rabbit antiserum

Against Au antigen standard. Two 3 kg rabbits were immunized with a standard Au antigen (Fig. 1) after first complexing the antigen in pooled stored sera by the following method. 2 ml of Au positive serum were added to an equivalent dose of human anti-Au IgG fraction in the presence of 0·02 M EDTA. The mixture was kept at 37°C for 4 hr and then 4°C for 48 hr. The precipitate of antigen–antibody complexes was collected by centrifugation at 6000 rpm for 30 min, washed three times in 0·02 M EDTA and twice in saline, and resuspended in saline to contain 1 mg/ml protein as determined by the method of Folin & Ciocalteu (1927). 2 ml of this immune complex was suspended in 2 ml of complete Freund's adjuvant and injected in multiple intramuscular sites. After 2 weeks, 2 ml of the complex with an equal volume of Freund's adjuvant were injected into multiple subcutaneous sites. One week later, the rabbits were given the first of three weekly immunizations of 1 ml of complex, first by the intramuscular, then subcutaneous, and finally intraperitoneal routes. The animals were bled 2 weeks after the last injection.

To 40 ml of the rabbit antiserum was added partially purified human IgG in order to remove antigammaglobulin antibodies. The mixture was kept at 37°C for 4 hr and 4°C for 48 hr. After centrifugation at 6000 rev/min for 30 min, the supernatant was collected and its

IgG fraction obtained by ammonium sulphate fractionation followed by DEAE-cellulose chromatography.

Control patients with Au antigenaemia. All the serum samples for each individual patient were pooled before reseparation into immunizing aliquots. Two rabbits weighing 3·0–3·5 kg, whose sera did not contain antihuman protein antibody, were immunized against the pooled sera of each of the five control patients (Fig. 1). The injections of 2 ml with an equal volume of Freund's adjuvant were made into the foot pads. After 2 weeks, the same material was injected into multiple subcutaneous sites. After another 2 weeks, weekly immunization with 1 ml of pooled serum was by three different routes alternating from intramuscular to intraperitoneal to subcutaneous sites. Only one cycle of this rotating schedule was actually carried out before immunization was discontinued, since the rabbits readily developed anti-Au antibody (Table 2).

TABLE 1. 'Australia antigen negative' patients with liver dysfunction

Patient	Sex	Age	Liver dysfunction		Heterologous anti-Au antibodies raised with sera
			Duration	Grade	
LD-176	F	13	2 weeks	+	No
LD-199	M	38	3 weeks	++	Yes
LD-209	M	38	9 weeks	+	No
LD-246	F	41	13 weeks	++	No
LD-262	F	11	4 months	+++	No
LD-263	F	28	3 months	+++	Yes
LD-264	F	41	3 weeks	+	No
LD-268	M	24	6 weeks	++	No
CD-17	F	17	32 months	+++	Yes
CD-34	F	19	14 months	+	Yes
CD-52	F	13	3 months	+	No

Liver dysfunction grade: +++, liver disease caused or contributed to death; ++, severe liver dysfunction; +, minor changes in liver function.

The sera collected from each pair of rabbits immunized against the sera of one of the five humans were absorbed with pooled serum from 100 normal human volunteers who were Au negative by the complement fixation technique. This step was designed to remove extraneous anti-human protein antibodies.

Patients without Au antigenaemia. Immunization of two rabbits was carried out with the pooled sera of each of the eleven patients (Fig. 1). The rotating injection schedule described in the previous section was continued for as long as 7 months or for whatever portion of that interval was required to develop heterologous anti-Au antibody.

The same absorption schedule as in the preceding section was carried out.

Negative controls. Aliquots of the pooled sera were used to immunize five rabbits for 6 months by the schedule described above, and the rabbit antisera were absorbed by the same methods.

TABLE 2. Reactions obtained by agar gel diffusion against standard Au antigen of antisera obtained from rabbits hyperimmunized with sera from 'Au negative' renal homograft recipients (Table 1) and Au positive controls

	Weeks after first immunization																											
Exp. no.	2	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
LD-176	–	–	–	N.T.	–	–	N.T.	–	–	–	N.T.	–	–	–	–	N.T.	–	–	–	–	–	–	N.T.	–	–	N.T.	–	–
LD-199	–	–	N.T.	–	–	–	N.T.	–	–	–	N.T.	+	+	+	E.D.													
LD-209	–	–	N.T.	–	–	–	N.T.	–	–	–	N.T.	–	–	–	–	N.T.	–	–	–	–	–	–	–	–	–	N.T.	–	–
LD-246	–	–	–	N.T.	–	–	N.T.	–	–	–	N.T.	–	–	–	–	N.T.	–	–	–	–	–	–	–	–	–	N.T.	–	–
LD-262	–	–	–	N.T.	–	–	–	N.T.	–	–	N.T.	–	–	–	–	N.T.	–	–	–	–	–	–	–	–	–	N.T.	–	–
LD-263	–	–	N.T.	–	–	–	–	N.T.	–	–	N.T.	–	–	–	–	N.T.	–	–	–	–	+	+	E.D.					
LD-264	–	–	–	N.T.	–	–	–	N.T.	–	–	N.T.	–	–	–	–	N.T.	–	–	–	–	–	–	–	–	–	–	–	–
LD-268	–	–	–	N.T.	–	–	–	N.T.	–	–	N.T.	–	–	–	–	N.T.	–	–	–	–	–	–	–	–	–	N.T.	–	–
CD-17	–	–	–	N.T.	–	–	–	N.T.	–	–	N.T.	–	–	–	–	N.T.	–	–	–	–	–	+	+	E.D.				
CD-34	–	–	N.T.	–	–	–	–	N.T.	–	–	N.T.	–	+	+	+	E.D.												
CD-52	–	–	N.T.	–	–	–	–	N.T.	–	–	N.T.	–	–	–	–	N.T.	–	–	–	–	–	–	–	–	–	N.T.	–	–
CONTROL GROUP*																												
1	+	+	+	E.D.																								
2	+	+	+	E.D.																								
3	+	+	+	E.D.																								
4	+	+	+	E.D.																								
5	+	+	+	E.D.																								

*Control group: 1, 2 and 3: sera of kidney transplantation patients positive for Au antigen by *in vitro* tests; 4 and 5: sera of patient with acute hepatitis containing Au antigen.

N.T., not tested; E.D., experiment discontinued; +, a single precipitin line was found by agar gel diffusion of rabbit antiserum with standard Au antigen positive sera.

RESULTS

Anti-Au activity of rabbit antisera

Raised against Au antigen standard. The rabbit IgG purified from antisera raised against immune complexes was tested on an Ouchterlony plate against the standard Au antigen sera and against the five Au positive controls. A single but broad precipitin line was formed (Fig. 1, plates A and B).

Raised against patients with Au antigenaemia. All five of the absorbed rabbit antisera raised against the sera of the Au positive control patients contained anti-Au activity when tested against any of these five control sera in the central Ouchterlony well (Fig. 1, plate B) or against the Au antigen standard sera (Fig. 1, plate A).

Raised against patients without Au antigenaemia. Seven of the eleven rabbit pairs immunized against Au negative pooled human sera failed to develop anti-Au antibodies despite immunization for as long as 7 months. However, anti-Au antibody was detected in the four other rabbit pairs 12, 15, 23 and 24 weeks after commencing immunization (Table 2). This activity was detected against the Au antigen standards (Fig. 1, plate A) as well as against all five of the control Au positive human sera (Fig. 1, plate B), and in each instance was manifested as a broad single precipitin line. However, the antisera raised by the four Australia negative sera did not react in any perceptible way with these four sera (Fig. 1, plate C) or with the seven others.

Negative controls. The rabbit antisera raised against pooled normal human serum did not possess any anti-Au activity (Fig. 1).

The identification of the antibodies

The broad single precipitin lines in the rabbit antisera raised against the Au negative human sera were identical to the precipitin lines produced by rabbit immunization with Au positive sera (Fig. 2), and fused with the much finer precipitin line formed by human anti-Au serum.

Clinical correlations

The eleven transplant patients with negative Au antigen by conventional techniques had the spectrum of liver disease shown in Table 1. The severity of hepatic dysfunction in the four patients whose sera raised anti-Au antibody in the rabbit was somewhat more severe than in the other seven patients, but both subgroups had variations from mild to fatal.

DISCUSSION

The primary subjects of this investigation were eleven patients with hepatic dysfunction following transplantation of the kidney in whom no evidence could be found of the hepatitis associated or Australia (Au) antigen by multiple tests including the highly sensitive complement fixation (Sever, 1962; Shulman & Barker, 1969) and HA or HAI (Vyas & Shulman, 1970) examinations. To unmask any examples of false Au negative, a 'biologic assay' was performed in which it was assumed that Au antigen was present in the sera of these patients and in sufficient quantities to hyperimmunize rabbits. The heterologous antisera raised against the sera of seven of the eleven patients did not support the foregoing premise in that no heterologous anti-Au antibodies ever became detectable.

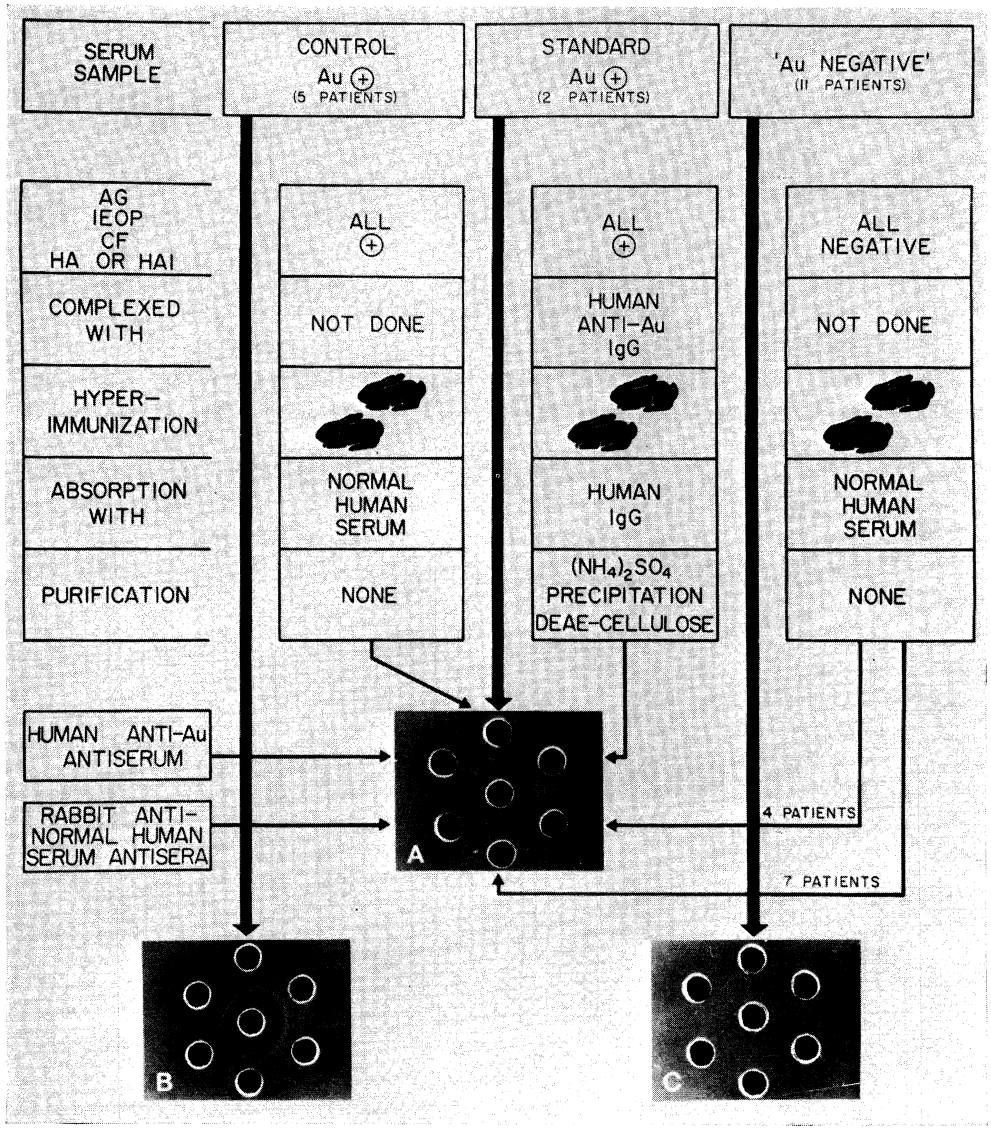

FIG. 1. Schematic representation of the methods and results. The upper portion of the figure diagrams the preparation of three kinds of rabbit antisera, each of which was then placed in a peripheral well of the agar plate, as indicated by the thin arrows. The fifth and sixth wells were filled with rabbit antisera to normal human serum and human anti-Au antiserum, respectively. The central well of plate A was charged with standard Au positive serum (broad arrow). The peripheral wells of the other two plates contain the same antisera in identical locations as plate A. However, the central wells were filled with control Au positive serum (plate B) or the serum from an 'Au negative' patient who by biologic assay had detectable Au antigen (plate C). Note the broad precipitin line in plates A and B and the absence of visible reaction in C.

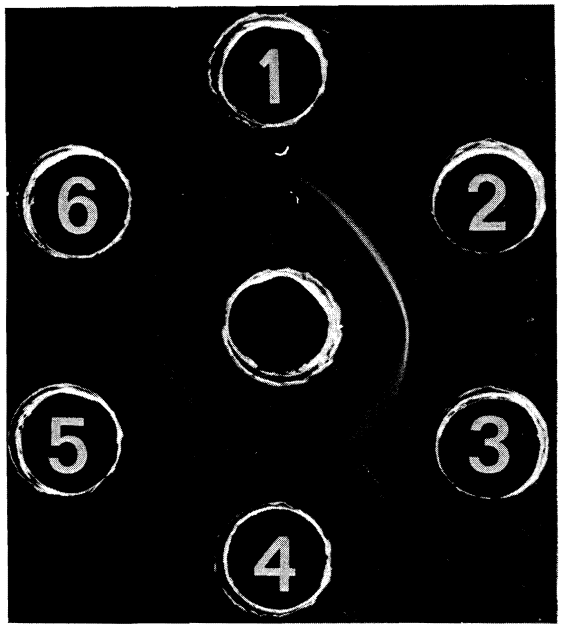

FIG. 2. Ouchterlony diffusion plate showing precipitin reactions of various antisera with the standard Au antigen (central well). Rabbit antisera raised against sera of patients CD-17, CD-34, LD-263 and LD-199, all thought to be 'Au negative,' were placed in peripheral wells 1, 3, 4 and 6, respectively. Well 2 was filled with human anti-Au antisera and well 5 with rabbit antiserum raised against standard Au antigen. All lines were identical, although the rabbit antisera produced broader precipitin bands than the human antiserum.

However, the pooled sera from each of the other four test patients did raise anti-Au antibodies in the rabbit which by agar gel diffusion were indistinguishable from those produced by immunizing with known Au positive human serum. These four patients, of whom two died primarily of liver complications, may have been suffering from serum hepatitis but with quantities of circulating Au antigen so minute that detection was not possible by any direct technique of examination. The indirect method of detection by rabbit immunization employing Freund's adjuvant and then testing against high titre Au standard serum was evidently not dependent on so high a level of antigen in the patient's sera. For epidemiologic purposes these four patients probably should be considered infectious carriers in the same sense as has been defined in other transplantation patients whose chronic Au antigenaemia could be identified with more conventional methods (Torisu *et al.*, 1971).

The evidence for Au antigen in these additional four patients with post-transplantation liver disease does not establish a cause-and-effect relationship for the hepatic malfunction as was stressed in a recent publication (Torisu *et al.*, 1971). Many immunosuppressed patients with chronic Au antigenaemia have no hepatic dysfunction whereas others, such as the majority of those in the present report, may have significant liver disease without any serologic findings whatever of serum hepatitis. In the latter patients or even in many Au

positive patients with hepatic malfunction, the principal cause of the liver findings could be hepatotoxicity of the immunosuppressive agents, particularly azathioprine. Support for this possibility has been provided by the prompt improvement in patients in whom azathioprine therapy was stopped because of abnormalities in liver function and replaced with cyclophosphamide (Torisu *et al.*, 1971; Starzl *et al.*, 1971).

The kind of study herein reported may be useful in investigating poorly understood hepatic diseases apart from those seen with immunosuppression and transplantation. An example is chronic aggressive hepatitis, a disorder that is associated with serum hepatitis and Au antigenaemia in about a quarter of the cases (Bulkley *et al.*, 1970; Vischer, 1970). It is probable that some or even many of the other three-quarters, said to be Au negative, also could be shown to be Au positive with the heterologous antiserum system.

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