Detection of Australia Antigen in Human Tissue Culture Preparations[1] (36048)

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Despite the fact that hepatitis is easily transmitted from one individual to another, innumerable attempts to isolate and culture a virus or other infectious agent from patients with hepatitis have met with limited success. There have been several reports of hepatitis production in humans from materials passaged in tissue culture and embryonated hen eggs (1–4). However, there has been no consistent and reproducible isolation, identification, or culture of the supposed infectious agent. Two major obstacles in this field have been the lack of laboratory means to detect and measure the presence of a hepatitis-producing agent without recourse to administering contaminated material to human volunteers, and the inability to transmit human hepatitis to experimental animals.

Australia antigen, Au(1), a particulate substance found in the blood of patients with hepatitis, has been implicated as a possible causative agent of human hepatitis (5). The present report describes our efforts to propagate Australia antigen in tissue culture systems.

Materials and Methods. In the first approach to this study Au(1) was added to line cells and primary cells in tissue culture. Hela (CCL 2), Detroit 6 (CCL 3), Intestine 407 (CCL 6) (from the American Type Culture Collection) and primary cells of liver, spleen, thymus, ovary, and skin from a 16-week-old human fetus (from Hospital of the University of Pennsylvania) were grown, harvested and inoculated with Au(1) positive sera and/or plasma or minced liver tissues from patients with Au(1) and hepatitis. Table I shows the total number of experiments conducted. The first 6 patients listed all had acute icteric hepatitis. The remainder were patients with Down's syndrome and chronic anicteric hepatitis. Control cultures were inoculated with either Au(1) negative sera and/or plasma or Earle's balanced salt solution. Monolayers 3–4 days on petri plates were exposed to the Au(1) inocula for 3 days and the medium was changed thereafter every 3–4 days. Slides or cover slips, placed in the plates initially for the purpose of staining with fluorescein coupled antisera by the method of Millman *et al.* (6), were removed periodically (up to 60 days). All culture fluids were concentrated by lyophilization and assayed for Au(1) by immunodiffusion and radioimmunoprecipitation assay (7, 8).

In the second approach, tissues from 131 different patients[3] were cultured (Table II). Forty-nine had Au(1) in their blood at the time the tissue was obtained and included patients with acute, subacute, and chronic hepatitis. The remainder were patients with a variety of hepatic and nonhepatic diseases who had liver biopsies for diagnostic purposes. Twenty-eight of the 68 specimens of washed liver biopsy material were cultured in flasks as finely minced material without trypsinization. Testis and jejunum specimens were minced and grown in flasks without prior

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[3] These specimens were obtained from the following hospitals in the Philadelphia area: the American Oncologic Hospital; Children's Hospital of Philadelphia; Jeanes Hospital; Jefferson Medical College Hospital; the Hospital of the University of Pennsylvania; the Philadelphia Veteran's Administration Hospital; and the Valley Forge General Hospital, Phoenixville, Pennsylvania.

TABLE I. Addition of Au(1) to Tissue Culture Monolayers.[a]

Each + = a separate experiment.

No.	Patient	Inoculum	HeLa	Det. 6	Int 407	Fetal					Bone marrow		Total no. of expts.
						Liver	Skin	Thymus	Ovary	Spleen	No. 1 D.J.	No. 2 R.Mc.	
1	Jas	Serum		+							+		2
2	Cit	Plasma				+				+			2
3	Hof	Plasma				++	+	+	+				5
4	Nel	Plasma				+							2
		Minced liver				+							
5	Blew	Plasma				+							1
6	Col	Serum	+	+									2
7	D.M.	Plasma				++							4
		Minced liver				++							
8	Fel	Serum			+		+						2
9	J.B.	Serum			+							+	2
10	Ada	Serum			+								1
11	E.B.	Serum			+								1
12	Cohi	Serum	+		+								2
13	Metz	Serum	+	+									2
14	Dona	Serum	+	+			+						3
	Total												31

[a] Cells grown in McCoy's medium (5a) (Grand Island Biological Company) with addition of potassium penicillin (83 units/ml), streptomycin sulfate (37 μg/ml), neomycin (37 μg/ml), and Mycostatin (50 units/ml). Fetal calf serum was added at a 30% (v/v) concentration at start and was reduced to 10% after establishment of monolayer.

TABLE II. Human Tissues Cultured for Australia Antigen.[a]

Tissue	How obtained	Total no. of patients	Patients with Au(1) (no.)	Length of time in culture (weeks)
Liver	64 Needle biopsy 4 Surgical biopsy	68	23	1–40
Sternal bone marrow	Needle aspiration	44	16	4–14
Testis	Surgical specimen	2	1	6–40
Jejunal loop	Surgical specimen	2	2	4–6
Lymphocytes	Peripheral blood	15	7	1
Total		131	49	

[a] See Table I for culture media. Cultures on polyethylene discs were grown in media containing 10% fetal calf serum without antibiotics. Gaseous environment was 5% CO_2 in air.

trypsinization. Sternal bone marrow material was inoculated directly into flasks containing medium. Lymphocytes were collected from the peripheral blood and cultured according to the method of Hungerford (9). Each lymphocyte specimen was cultured in duplicate with, and without, the addition of phytohemagglutinin. All tissue culture fluids were saved, concentrated by lyophilization 10-fold, and stored frozen until ready for assay.

Forty of the 68 liver specimens were trypsinized, minced, and cultured on polyethylene discs by the method of Taylor *et al.* (10). Four to 6 implants were made from each specimen. The implants from each biopsy were placed in separate culture plates containing wells. Each disc was floated, cell surface down, over medium. The discs were removed at various intervals and stained with acridine orange; and the fluids were saved

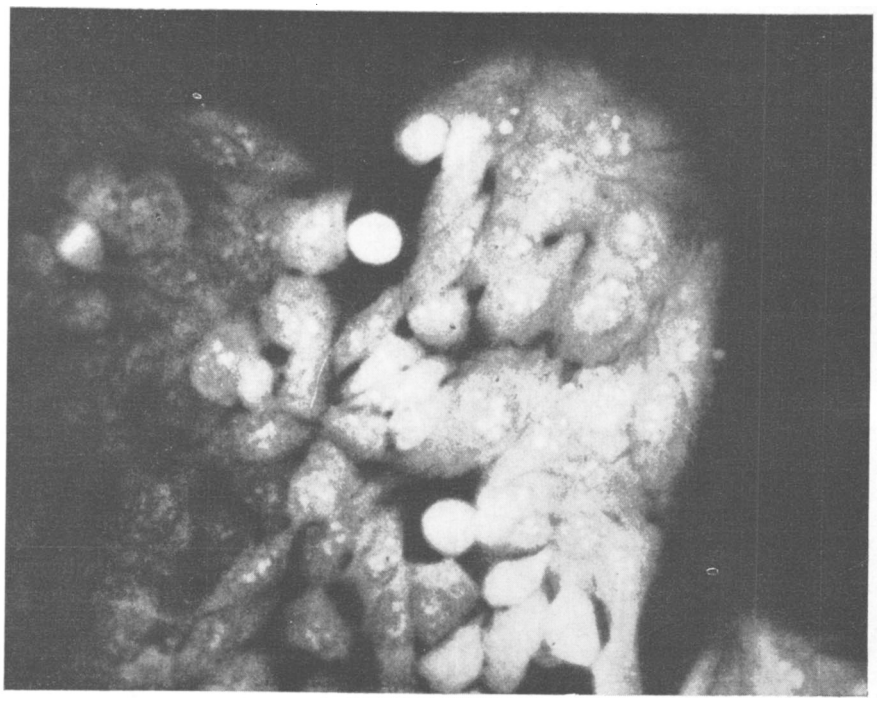

FIG. 1. Human liver (L.H.) cultured in flasks and then subcultured on polyethylene discs by the method of Taylor *et al.* (10): These cells, from the sixth passage, were stained with acridine orange; ×1250.

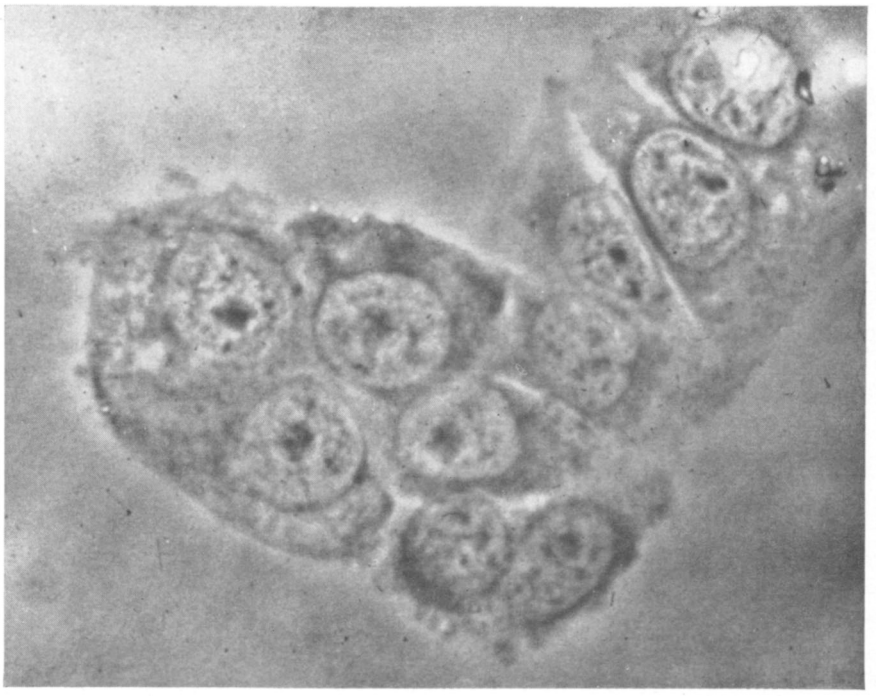

FIG. 2. Human liver (L.H.) cultured on microscope slides in petri plates: Cells are from day 5 in culture from passage two. Phase contrast; ×2250.

for Au(1) assay.

Results. Of the 28 liver biopsy specimens cultured in flasks, one specimen produced an epitheliod cell type culture which is shown in Fig. 1. This biopsy was from a patient who had acute, icteric hepatitis and Au(1) in his blood at the time the biopsy was performed. Fluorescent staining of the original biopsy specimen showed fluorescent granules in the cell nuclei (6). The culture obtained from this biopsy has been propagated through 16 passages without obvious microscopic change in the appearance of the cells. The results of assays for Au(1) are shown in Table III. Fluorescent granules were seen in the nuclei on days 5, 6, 7, 8, and 11 of the second passage. With further culturing, the fluorescent granules were no longer seen until passage 6, when weakly positive fluorescent granules were again demonstrated in the cell nuclei. None of these culture fluids proved positive by immunodiffusion assay. Cells from passage 6 were also grown on 32 polyethylene discs. Each disc was transferred to a new well with fresh medium every 3–4 days. When the discs became crowded they were trypsinized and new cultures were started. Fluids from 2 of the 32 discs contained Au(1) as determined by radioimmunoassay (7, 8); one on day 8 of passage 6 after 113 days in culture and another on day 33 from the same passage after 139 days in culture. Neither culture fluid proved positive by immunodiffusion assay. Both of these assays were greater than 2 standard deviations from the mean of 4 Au(1) negative sera used as controls in this assay.[4] Figures 2 and 3 show the appearance of these liver cells in phase and UV light after staining with fluorescein coupled rabbit antibody. These cells are from day 5 in culture from passage 2. Cells grown on polyethylene discs

[4] This test is so designed that a known amount of radioactive ^{125}I-tagged Au(1) is mixed with an amount of antibody to Au(1) that will precipitate 50% of the radioactivity. When a test specimen is added to the system, a depression in the amount of precipitated radioactivity indicates that the test sample contains Au(1) which is competing with the radioactive Au(1) for antibody.

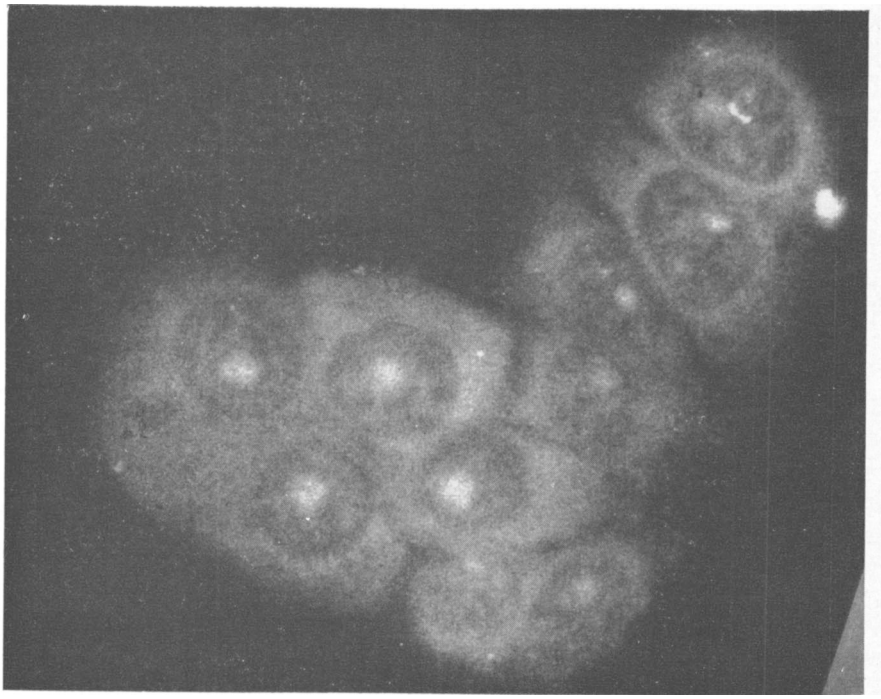

FIG. 3. Same as Fig. 2 but stained with fluorescein isothiocyanate coupled rabbit antiserum for Australia antigen. Bright areas within nuclei stain specifically green while cytoplasm shows autofluorescent grey blue; ×2250.

could not be stained with fluorescein due to the retention of the stain by the polyethylene discs.

All of the 40 liver biopsy specimens that were cultured directly on polyethylene discs produced viable cells and several cell divisions for at least 8 days as observed by staining with acridine orange. Tissue culture fluids derived from 1 specimen were found to contain Au(1) by radioimmunoassay. This specimen was from a patient who had both sarcoidosis with granulomatous changes in the liver and a primary hepatoma. Tissue from the hepatoma and that part of the liver involved with sarcoidosis were cultured separately. Three discs (out of a total of 14 made from the sarcoid involved liver) only contained Au(1) by radioimmunoassay (Table IV). Au(1) appeared in the tissue culture fluids in 6, 20, and 26 days.

None of the other primary tissues studied gave evidence of Au(1) either in the cells or the tissue culture fluids.

All attempts to propagate Au(1) by inoculating tissue culture monolayers (Table I) with Au(1) containing materials failed. Variations including changes in temperature, prior exposure of the cells to UV light, or the addition of adenovirus type 12 as helper virus had no measurable effect within the limits of these experiments.

Discussion. These results appear to confirm our earlier report (6) that Au(1) may be present in the liver of patients who have hepatitis and Au(1) in their blood. The appearance of fluorescent granules in the cell culture of L.H. after 6 passages indicates that either Au(1) is being carried from one cell generation to another laterally or that it is being transmitted vertically and is replicated by the cells. The fact that the fluorescent granules were not observed persistently throughout the cultures would tend to favor the latter explanation. The failure to find Au(1) in the fluids of the second passage from patient L.H. (Table III) may indicate that Au(1) is firmly bound to the cells and not easily released in detectable amount.

Positive results were found with only 2 liver cultures out of 23 specimens from pa-

TABLE III. Results of Experiments with Culture from Liver of L.H.

Total time in culture[a] (days)	Time in passage no. 2 (days)	Detection of Au(1) by: Fluorescence	Immunodiffusion	RIP
A. Cells from passage no. 2 grown in petri plates				
58	1	Neg	Neg	Neg
61	4	Neg	Neg	Neg
62	5	Pos	Neg	Neg
63	6	Pos	Neg	Neg
64	7	Pos	Neg	Neg
65	8	Pos	Neg	Neg
68	11	Weakly pos	Neg	Neg
B. Cells from passage no. 6 grown on discs				(SD)[b]
113	(Disc no. 2) 8		Neg	Pos (>2)
139	(Disc no. 6) 33		Neg	Pos (>3)

[a] Calculated from date of biopsy.

[b] Radioimmunoprecipitation assay and number of standard deviations. Values greater than 2 SD from the mean of the controls [negative Au(1) sera] are considered significant.

tients with Au(1) in their blood. This could be entirely due to the difficulties of maintaining primary liver cells in culture. More of these specimens might have proved positive had they been maintained in culture longer. On the other hand the 2 specimens of liver that produced positive results might have been somewhat altered *in vivo* making *in vitro* cultivation possible. One specimen was from an individual with acute icteric hepatitis, whose liver cells displayed fluorescent intranuclear granulation at the time of biopsy and the other was from an individual with sarcoidosis and hepatoma.

The results reported here are compatible with the hypothesis that Au(1) can be passaged in liver cells in tissue culture. In addition it is highly probable that an increase in Au(1) has resulted from replication *in vitro*.

TABLE IV. Radioimmunoprecipitation Assay of Tissue Culture Fluids from Patient H.A.

Time in culture (days)	Au(1)	SD[a]
5	Weakly pos	(2)
6	Pos	(>6)
20	Pos	(>6)
26	Pos	(>6)
40	Weakly pos	(2)

[a] Values greater than 2 SD of the mean of the controls [negative Au(1) sera] are considered significant.

Summary. An attempt was made to propagate Australia antigen, Au(1), in tissue culture. The approach to the study was twofold: (a) the culturing of fresh biopsied tissues from patients with hepatitis and Au(1) in their blood; and (b) the addition of serum, plasma, and extracts of biopsied liver from patients with Au(1) in their blood to established cell lines and primary cells from human fetal tissues in tissue culture. Positive results were obtained only with the first approach. Two liver cultures out of 23 specimens from patients with Au(1) in their blood produced either intranuclear fluorescent granules after staining with fluorescent coupled rabbit anti-Au(1) antiserum or Au(1) in the tissue culture fluids as determined by a sensitive radioimmunoprecipitation assay technique. Fluorescent intranuclear granulation appeared during the second and sixth passage of one culture of liver. It is unlikely that this could be explained by carry-over of Au(1) from the initial biopsy specimen. Cultures of sternal bone marrow, testis, jejunal loop, and lymphocytes from patients who had Au(1) in their blood were uniformly negative for fluorescent granules as well as Au(1) by radioimmunoprecipitation assay. The results indicate a strong possibility that Au(1) replicates in

tissue culture.

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