Immunoepidemiological and in Vitro Studies of Possible Relationships between Australia Antigen and Hepatocellular Carcinoma

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SUMMARY

The prevalence of Australia antigen [Au(1)] in sera of patients with hepatocellular carcinoma was lower than in the blood donor population at The University College Hospital, Ibadan, Nigeria, but that of the antibody was higher. Only one patient (2%) had Au(1) but two (4%) had the antibody in 50 liver cell carcinoma cases proved either by biopsy or at autopsy. Sixty-eight % were α-fetoprotein (AFP) positive, and the only positive tumor for Au(1) was also AFP positive. Immunofluorescent tests on some liver tissue from those carcinomas showed no Au(1) particles in either the nucleus or the cytoplasm, whereas they were present in liver biopsies from carriers of Au(1). Cultured cells from liver biopsies of patients with various diseases fed with media containing 20% human sera with Au(1) showed nuclear and cytoplasmic fluorescent particles after 72 hr by direct immunofluorescence. One week afterward, the cells from hepatocellular carcinoma began to show cytopathic effects and all were nonviable 24 hr later. Control cultures were still doing well 6 months later. No malignant transformation or cellular retrodifferentiation of any of the cultures was observed. However, cultures fed 2-acetylaminofluorene and tannic acid did show conversion to AFP-positive cells, interpreted as malignant transformation, after 70 days. Transplantation to cortisone-treated and irradiated rats resulted in continued production of human AFP. All of the cultured cells had diploid (46) chromosomes, but there appeared to be more chromatid breaks in the cells treated with Au(1) and carcinogens than there were in the controls. The cells transformed by the chemical carcinogens also showed cytopathic effects after 7 days of feeding with serum containing Au(1). Lymphocytes of patients with hepatocellular carcinoma and cirrhosis of the liver showed a higher percentage of blast transformation with Au(1)-containing sera than did the lymphocytes of Au(1)-negative blood donors and of patients with chronic duodenal ulceration and amoebic liver abscess.

INTRODUCTION

A number of reports have associated a high prevalence of Australia antigen [Au(1)] with hepatocellular carcinoma, including those of Prince (28), Vogel et al. (47), Bagshawe et al. (3), Velasco et al. (46), and Teres et al. (44). However, Simons et al. (35) from Singapore and Smith and Blumberg (40) from Uganda did not find a higher prevalence of Au(1) in primary liver cell carcinoma. Our pilot results were similar to those of Simons et al. (35) and of Smith and Blumberg (40). Experiments were performed to shed more light on this relationship. We had also observed, in a preliminary trial, that Au(1)-containing sera stimulates blastic transformation of lymphocytes from patients known to have had hepatitis seropositive for Au(1) but who had become negative for Au(1) at the time of testing. This implied that a past infection by Au(1) could be diagnosed by this technique. We therefore decided to apply this technique to the investigation of patients with cirrhosis of the liver and hepatocellular carcinoma. Having observed in our preliminary experiments that Au(1)-containing serum is cytopathic on some cultures from human hepatocellular carcinoma, we wanted to find out whether this phenomenon could be used to detect malignant transformation or cellular retrodifferentiation of human liver cells in cultures treated with chemical carcinogens. Other criteria for cancer tested included the secretion of AFP, transplantability of the cancer in irradiated and cortisone-treated rats, and chromosomal alterations.

MATERIALS AND METHODS

Epidemiology Section

Serology

Sera from 50 patients with hepatocellular carcinoma that had been diagnosed by biopsy and/or autopsy were tested by double diffusion and electroosmodiffusion for Au(1) and its antibody, according to the methods of Prince (27) and Bedariga et al. (4). These sera were also tested for AFP according to the method of Smith et al. (36). Portions of liver biopsies from 6 of these patients were snap frozen in liquid nitrogen (−70°) and stored at −70° in a Revco Ultra-low freezer, until needed. Other portions were cultured in ring chambers according to the method of Smith et al. (37).

1 The abbreviations used are: AFP, α-fetoprotein; PHA, phytohemagglutinin; TC 199, tissue culture Medium 199; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; AAF, 2-acetylaminofluorene.
Sera were collected from 15 patients with cirrhosis of the liver, diagnosed clinically. Liver biopsies were performed on 12 of these patients and were treated as for patients with primary liver carcinoma. Sera and liver tissue were obtained from 20 patients with various diseases, including 12 patients with chronic duodenal ulceration, 3 with amoebic liver abscess, and 5 with chronic hepatitis. These served as controls.

**Lymphocyte Transformation**

For this test, 30 ml of blood were obtained from each patient and added to 210 units of phenol-free heparin in a syringe, and then were mixed with 5 ml of sterile 1.5% dextran and allowed to stand at room temperature for 2 hr. From each sample, the following tests were set up. (a) Two ml of serum were mixed with 6 drops of PHA, and the final volume was made up to 10 ml with TC 199. (b) Two ml of serum were mixed with 2 ml of serum that was known to contain Au(1) and that had been passed through a Millipore filter to remove all formed elements of blood and bacteria. The final volume was made up to 10 ml with TC 199. (c) Two ml of serum were made up to 10 ml with TC 199. These were then incubated at 37° for 72 hr. Then each sample was centrifuged, and from the deposit 9 thin smears were made on clean glass slides which were then air dried, fixed in methyl alcohol, and stained with May-Grünwald-Giemsa for scoring on a morphological basis, according to the method of Holborow and Johnson (16). This test was performed on the following patients: 10 with hepatocellular carcinoma, 12 with cirrhosis of the liver, 12 with chronic duodenal ulceration, 3 with amoebic liver abscess, and 5 with chronic hepatitis; it was also performed on 10 normal blood donors seronegative for Au(1) and 10 blood donors seropositive for Au(1).

**Indirect Immunofluorescence.** This test was performed on 4-μm cryostat sections of the snap-frozen liver biopsies, dried with a hair drier, and on cultures of the liver biopsies. Rabbit anti-Au(1) conjugated with FITC, prepared according to the method of Melartin and Blumberg (21), was used. The conjugate was tested for specificity by the Ouchterlony technique according to the method of Prince (27), with reference reagents from several laboratories including those of Dr. A. J. Zuckerman (Poland), Dr. K. Okochi (Japan), Dr. A. J. Zuckerman (United Kingdom), Dr. S. Cunningham (United States), and Dr. B. S. Blumberg (United States).

The specificity of the fluorescence was confirmed by treating controls with unconjugated monospecific rabbit antiserum to Au(1) before adding the conjugate. No fluorescence was observed. Some of the conjugate was also absorbed with human liver powder obtained at autopsy from a patient seropositive for Au(1). This powder was prepared according to the method of Holborow and Johnson (16). Sections treated with these absorbed conjugates also did not show any fluorescence.

**Direct Immunofluorescence.** This test was done on 4-μm cryostat sections of the snap-frozen liver biopsies, dried with a hair drier, and on cultures of the liver biopsies. Rabbit anti-Au(1) conjugated with FITC, prepared according to the method of Melartin and Blumberg (21), was used. The conjugate was tested for specificity by the Ouchterlony technique according to the method of Prince (27), with reference reagents from several laboratories including those of Dr. A. J. Zuckerman (Poland), Dr. K. Okochi (Japan), Dr. A. J. Zuckerman (United Kingdom), Dr. S. Cunningham (United States), and Dr. B. S. Blumberg (United States).

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harvested slides were washed with PBS for 2 hr and dried overnight at 25° before being used for immunofluorescent studies. These were simple explant cultures, although some separated cells did adhere to glass also, and they were never subcultured before use in any of the described experiments. Enzyme histochemistry for uridine diphosphoglucose glycogen synthetase, ATPase acid and alkaline phosphatase, and 5-nucleotidase and naphthylamidase; special stains for glycogen, bile, and lipofuscin; and electron microscopy, as well as reactions to adenoviruses 5 and 7, indicate that the cells that grow from liver biopsy include hepatocytes, fibroblasts, endothelial cells, and probably bile duct cells and Kupffer cells (9, 10, 51, 52).

Twenty-seven ring chambers (0.8-ml capacity) and 10 medicine bottles (150-ml capacity) of liver biopsy cultures from patients with chronic duodenal ulcer each were fed with 0.26% AAF, a chemical carcinogen (7, 11, 48) dissolved in 1 drop of dimethylformamide and 5 ml TC 199. To the ring chamber 1 drop of this was applied to 6 drops of complete medium, consisting of TC 199, 20% normal human sera without Au(I), 1% chicken embryo extract, 33 units of neomycin per ml, and 125 units of mycostatin per ml. One drop of 0.8% tannic acid, another chemical carcinogen (19, 22), was added every 3 days for 4 doses. However, the AAF was added with fresh medium every 3 days for the duration of this experiment, which continued 70 days. Equivalent amounts of these carcinogens were added to the bottle cultures. The cells from each chamber, which numbered about 3 × 10⁵, were periodically injected i.p. into 30-g rats that were irradiated with 250 rads γ-rays and that received 2.5 mg hydrocortisone acetate B.P. s.c. daily for 3 days. The injection was done on the 4th day. Supernatants from each of the chambers were pooled and tested every day, after being concentrated 100 times by ultrafiltration at 4°, for human AFP. The complete medium with Au(I) was prepared with a pool of 3 sera from normal blood donors known to contain Au(I) and was substituted for 20% normal human serum. Control cultures were not treated with the carcinogens. On Day 70, some cultures were fed with Au(I), some were preserved for immunofluorescence to AFP, and others were prepared for chromosome analysis, as had been done periodically before.

**RESULTS**

**Epidemiology Section**

**Serology and Immunofluorescence**

One (2%) of the 50 patients with hepatocellular carcinoma had Au(I) in the serum and 2 (4%) had the antibody. Thirty-four patients (68%) were AFP-positive. The only Au(I)-positive hepatocellular carcinoma was also AFP positive. The 2 liver cancer patients with Au(I) antibody were AFP negative. Liver biopsy could be done on 6 patients with hepatocellular carcinoma, all of whom were seronegative for Au(I). Direct immunofluorescence with FITC-conjugated rabbit anti-Au(I) did not show any fluorescence in these biopsies, but nuclear and cytoplasmic fluorescent particles were seen in 11 biopsies from normal blood donors seropositive for Au(I) performed in another study. This is reported in detail elsewhere. None of the sera of the 12 cirrhotic patients, 12 chronic duodenal ulcer patients, 3 patients with amoebic liver abscess, or of the 5 patients with chronic hepatitis was Au(I) seropositive. The direct immunofluorescent test was also negative on the liver biopsies from these patients.

<table>
<thead>
<tr>
<th>Test</th>
<th>Hepatocellular carcinoma (10)</th>
<th>Cirrhosis of the liver (12)</th>
<th>Chronic hepatitis (5)</th>
<th>Chronic duodenal ulcer (12)</th>
<th>Amoebic liver abscess (3)</th>
<th>AU(I) + (10)</th>
<th>AU(I) – (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>0.70 ± 0.5</td>
<td>0.6 ± 0.4</td>
<td>0.7 ± 0.6</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>1.0 ± 0.5</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>PHA</td>
<td>70.2 ± 8.0</td>
<td>68.5 ± 8.6</td>
<td>60.3 ± 9.1</td>
<td>75.0 ± 10.2</td>
<td>67.5 ± 3.7</td>
<td>73.1 ± 8.0</td>
<td>70.1 ± 9.0</td>
</tr>
<tr>
<td>Au(I)</td>
<td>8.0 ± 3.2</td>
<td>5.2 ± 4.1</td>
<td>2.1 ± 0.9</td>
<td>1.1 ± 0.6c</td>
<td>0.9 ± 0.3</td>
<td>1.2 ± 0.7</td>
<td>2.0 ± 1.0</td>
</tr>
</tbody>
</table>

- The figures in parentheses, number of patients tested in each group.
- +, positive; –, negative.
- c t = 5.994; p < 0.001.
 AFP in the rat serum within 7 days. On the 70th day, the brown granular cells started to migrate away from the ulceration fed with AAF and tannic acid were morphologically which were still rich in cells even up to 6 months later, cortisone-treated rats did not result in detection of human AFP. Seven days later, the cultures of hepatocellular carcinoma began to show a cytopathic effect, such as the rounding up and withdrawal of the cytoplasmic projections under phase-contrast microscopy. Twenty-four hr later, no more cells were seen sticking to glass. On washing and staining, no cells were seen, compared with the other cultures which were still rich in cells even up to 6 months later, including cultures from hepatocellular carcinoma fed with complete medium without Au(1). Studies are in progress to see whether Au(1) can be passaged from the supernatants and also to estimate its quantitative changes.

**Lymphocyte Transformation**

Whereas PHA transformed a large proportion of the lymphocytes to blasts in all of the groups, Au(1)-containing serum caused a statistically significant stimulation of the lymphocytes only of those patients with hepatocellular carcinoma and cirrhosis of the liver, compared both with patients with chronic duodenal ulcer and amoebic liver abscess and with blood donors (Table 1).

**Tissue Culture Section**

**Effect of Au(1) on Cultured Cells from Liver Biopsies**

Cultured liver biopsy cells fed with complete medium containing 20% human serum from a pool of 3 sera with Au(1) did not show any morphological alteration with the phase-contrast microscope for the 6-month period of observation. The medium was changed every 3 days. However, 72 hr after we started the culture, when cells started to grow very well from the explants, cytoplasmic and some nuclear fluorescent particles were demonstrable by the direct immunofluorescent technique. These cultured cells were originally negative for Au(1), since controls of cultures from the same biopsy not fed with Au(1) were negative from Day 1 through 3. In the hepatocellular carcinoma cultures, fluorescent particles were seen in 24 hr, when many cells were already sprouting from the explants (Figs. 1 and 2). When the liver specimens were first cultured with complete medium without Au(1) for 1 week before feeding with Au(1) began, the antigen was demonstrable in the nucleus and cytoplasm of cultures of hepatocellular carcinoma in 6 hr, but it took 48 hr for the effect to be observed in liver cultures from biopsies of patients with other diseases. Seven days later, the cultures of hepatocellular carcinoma began to show a cytopathic effect, such as the rounding up and withdrawal of the cytoplasmic projections under phase-contrast microscopy. Twenty-four hr later, no more cells were seen sticking to glass. On washing and staining, no cells were seen, compared with the other cultures which were still rich in cells even up to 6 months later, including cultures from hepatocellular carcinoma fed with complete medium without Au(1). Studies are in progress to see whether Au(1) can be passaged from the supernatants and also to estimate its quantitative changes.

**Chromosome Analysis**

This was done on the cultured cells at intervals and showed that most of the cells were diploid, with occasional tetraploid cells. The striking feature was the marked increase in chromatid breaks in the cells treated with Au(1) and carciogen, compared with controls.

**DISCUSSION**

It has long been postulated that viral hepatitis could be a precursor of postnecrotic cirrhosis, which is associated with hepatocellular carcinoma in the tropics. With the discovery of AFP and Australia antigen, Smith and Blumberg (40) remarked that these could be exploited as markers in testing the hypothesis that viral hepatitis sometimes leads to cirrhosis of the liver which then undergoes malignant change into hepatocellular carcinoma.

There have been recent reports on the prevalence of Au(1) in hepatocellular carcinoma from different parts of the world (3, 28, 47). Most authors record a very high incidence of Au(1); however, there have been 2 reports (35, 40) of a low incidence of Au(1) in hepatocellular carcinoma. This low prevalence is also found in Ibadan, Nigeria. We found a prevalence of 5.1% for Au(1) and 0.13% for the antibody in 2045 blood donors (38). In 423 school children, we found an incidence of 6.7% for the antigen and 0.02% for the antibody (13). In 50 patients with hepatocellular carcinoma, the prevalence was 2% for Au(1) but was 4% for the antibody. This rather high antibody rate, which indicates previous exposure to Au(1) antigen, if added to that of antigen in the determination of the prevalence rate, will result in its increase to 6%. This would make the prevalence of Au(1) in liver cell carcinoma the same as that of the blood donor population. This result is still very different from those of workers in Dakar and Uganda, where a prevalence of about 40% for Au(1) occurred in hepatocellular carcinoma compared with 6% in blood donors.

Several reports have indicated the relatively high incidence of positive AFP in hepatocellular carcinoma in areas of high incidence of the disease. There appears to be a geographic variation in this incidence also (12, 25). The finding of a 68% positivity rate for AFP in this study is in close agreement with
that of 70% reported in the collaborative study of O'Connor et al. (25) on sera from this same locality, although from different patients. The prevalence of Au(1) in those sera, if still available, should be instructive. The relationship between Au(1) and AFP remains to be clarified. Some liver cell carcinomas with Au(1) are also AFP-positive. This is in contrast to liver cell carcinomas induced in rats with aflatoxin which are all AFP negative (41).

It has been demonstrated that AFP is produced by cultured cells. Irlin et al. (18) used tissue culture cells of transplantable ascitic hepatoma and double diffusion. Alevbev and Bakirov (2) demonstrated AFP in the livers of newborn mice and rats with the aid of immunoautoradiography. Gitlin and Boesman (15), using the latter technique, also showed the presence of AFP in the supernatant fluids of some cultured human and rat fetal tissues. Luria et al. (20), using organ culture of embryonal mouse liver, and Van Furth and Adinolfi (45), using human fetal livers in vitro and double diffusion plus immunoautoradiography, confirmed these observations. Smith et al. (37) extended these observations by demonstrating AFP with immunofluorescence on cultured human hepatocellular carcinoma cells. These studies were on fetal or neoplastic tissue. Secretion of AFP tends to diminish, and sometimes to stop, after prolonged cultivation of malignant hepatomas (18). We have shown that adult, differentiated liver cells which were at first not secreting AFP detectable by double diffusion or immunofluorescence, after treatment with AAF and tannic acid, which are known chemical hepatocarcinogens, resumed the secretion of AFP after 70 days of this treatment. It is known that AAF needs to be activated in vivo in the liver before becoming effective as a carcinogen (8, 34). If the observed change in vitro is interpreted as a malignant change, then the hepatocytes among the cultured cells might have effected the necessary activation.

The presence of AFP in cell culture supernatant fluid as a marker for malignant transformation might be difficult to accept. Abelev (1), Ruoslahti and Seppala (32, 33), and Purves and Geddes (30) have reported finding AFP in normal human sera by the radioimmunoassay technique. Nishi (24) and Bernades et al. (5) have reported finding serum AFP in metastatic tumors of the liver by the Ouchterlony technique. Also, Jeffroy et al. (14) and Smith (39) have reported the presence of serum AFP in acute viral hepatitis by immunoprecipitation and immunoelectrophoresis, respectively. It is now known that it is the quantity of AFP present that is significant, since radioimmunoassay is about 1000 times more sensitive than immunodiffusion, which seems to pick up the high levels of AFP present in the fetus and in cancer. It may be that what has been observed by treatment with the chemical hepatocarcinogens is an in vitro cell retrodifferentiation rather than an in vitro cellular malignant transformation. The cytogenetic findings would support this, and the heterologous transplantation experiment might also indicate that fetal tissues are transplantable up to a stage.

The use of in vitro methods and cytogenetics in the study of human cancers is well established (17, 23, 29, 31). Brighton et al. (6) have observed the effect of Au(1)-containing serum on differentiated liver cells in cultures, and our findings confirm their results. The cytotoxic effect of Au(1)-containing human sera on cultured hepatocellular carcinomas seronegative for Au(1), and on cultured human liver cells transformed by chemical carcinogens, indicates a differential susceptibility to this agent of human malignant hepatocytes and of normal liver cells. It also demonstrates that the neoplastic liver is as susceptible to Au(1), if not more susceptible than normal liver. It further suggests that the mechanism of action of Au(1) is by means of a direct necrogenic effect on cells rather than an indirect action mediated by immunological means. It would be fascinating to know whether this cytopathic effect can occur when Au(1)-containing serum is inoculated into other human and experimental cancers in vitro. Cultures of liver biopsies of carriers of Au(1) grow well in vitro. We do not know whether biopsies of hepatocellular carcinomas with Au(1) will grow and, if they do grow, whether they will survive for over 1 week.

The predominantly diploid karyotype of embryonal hepatocytes in culture has been previously reported (53). It was rather surprising that in neoplasia these cells still retained this characteristic, although there were more chromatid breaks present as well. No marker chromosome was seen, and the usually wide scatter of karyotypes from the hypodiploid to hyperdiploid types was absent. It is possible that not all of the cells in the culture from biopsies of hepatocellular carcinomas were malignant. Although highly unlikely, the few malignant cells present might not have been in division at the time of the chromosome preparation.

Yeung-Laiwah (49) reported on the probable specificity of lymphocyte transformation by serum rich in Au(1) in detecting past infections with the agent. This communication confirms that observation and extends it in revealing that, when the serum is seronegative for Au(1) in hepatocellular carcinoma, sensitized lymphocytes could be transformed by the antigen because they had been exposed to it sometimes in the past. Although this does not prove that hepatocellular carcinoma is caused by Au(1), it does imply that the liver with hepatocellular carcinoma had at some time in the past been exposed to Au(1). This could have occurred before or after the malignant change in the organ. Whereas we believe that the etiology of hepatocellular carcinoma in the tropics is probably multifactorial, the role of Au(1) in its pathogenesis can be firmly resolved by testing the sera of children regularly for Au(1) as well as for AFP, and by following them up for a long period, if feasible, to see how many of the children with Au(1) eventually become AFP positive, with histological evidence of hepatocellular carcinoma.

All the changes ascribed to Au(1) in the described experiments have been associated with the use of sera containing the antigen. Although these experiments are reproducible, some having been repeated up to 6 times with different batches of Au(1)-containing sera, it is definitely possible that these changes occurring in the presence of Au(1)-containing sera are not due to Au(1) alone. In spite of the controls and the specificity of the antisera used, there may be other unknown factors in the Au(1)-containing sera that have contributed to the observed effects. We hope to clarify this difficult point when pure Au(1) becomes available to us and when we succeed in culturing normal and neoplastic hepatocytes in chemically defined, serum-free, synthetic...
REFERENCES

ACKNOWLEDGMENTS

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Fig. 1. Cells, cultured 36 hr, from needle liver biopsy on a patient with hepatocellular carcinoma that was both AFP and Au(I) seronegative. Direct immunofluorescence for Au(I). Culture fed for 36 hr with medium containing Au(I) when this photograph was taken. Diffuse nuclear fluorescence in a few round cells. × 280.

Fig. 2. Cells, cultured 36 hr, from needle liver biopsy on a patient with hepatocellular carcinoma that was both AFP and Au(I) seronegative. Direct immunofluorescence for Au(I). Culture fed for 36 hr with medium containing Au(I) when this photograph was taken. Coarsely granular cytoplasmic fluorescent particles. × 1260.
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