PAPERS AND ORIGINALS

Specific Immunodiagnosis of Hepatocellular Carcinoma by Leucocyte Adherence Inhibition

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British Medical Journal, 1974, 2, 349-352

Summary

The leucocyte adherence inhibition test provides a rapid, reliable, and specific technique for the immunodiagnosis of primary hepatocellular carcinoma (malignant hepatoma). The patient's blood leucocytes are tested in vitro for cell-mediated immunity against tumour-associated antigens and the serum is tested for blocking factor which interferes with the immunological reaction. Specific reactivity of both leucocytes and serum was consistently detected in patients with malignant hepatoma, and negative reactions were obtained in other liver diseases including secondary tumours of the liver. The test has provided positive evidence for the presence of hepatoma when more conventional methods gave doubtful or negative results. A positive result preceded the clinical appearance of tumour by up to three years in two patients.

Introduction

The early diagnosis of primary hepatocellular carcinoma (malignant hepatoma) poses several difficulties for the clinician. Hepatomas are often preceded by other pathological conditions of the liver, such as cirrhosis and haemochromatosis (Finch and Finch, 1955), so that a change in symptoms due to the tumour may escape recognition. A small tumour may not be detected by hepatic scanning, biopsy, or angiography. Moreover, currently available serological tests lack specificity and sensitivity.

Possible immunological aids to the diagnosis of primary liver cancer include detection of α -fetoprotein in the serum, demonstration of a specific tissue carcinofetal ferritin (Alpert *et al.*, 1973), and examination of leucocytes for cell-mediated immunity against tumour-associated antigens. All of these have practical limitations as performed at present. α -Fetoprotein was found in less than 30% of the sera from patients of Caucasian origin with hepatoma, the proportion being greater in Africans (Stillman and Zamcheck, 1970). Tests for lymphocyte cytotoxicity (Hellström *et al.*, 1971 a; Jagarlamoodu *et al.*, 1971), lymphocyte transformation (Vanky *et al.*, 1971), and inhibition of leucocyte migration (Andersen *et al.*, 1969; Segall *et al.*, 1972) are applicable to the detection of specific antitumour cell-mediated immunity in a variety of cancers and are potentially applicable to hepatoma; these, however, are complex techniques.

Many and perhaps all human cancers induce the appearance of cytotoxic host lymphocytes capable of killing cultivated tumour cells. This activity is accompanied by circulating blocking factors which interfere with cell-mediated immunity in vitro (Hellström *et al.*, 1971 a; Jagarlamoodu *et al.*, 1971; Vanky *et al.*, 1971) and are probably essential for tumour progression in vivo (Hellström *et al.*, 1971 a, b; Hellström and Hellström, 1973). Previously described methods for the detection of these cellular and humoral entities require elaborate, time-consuming procedures.

The leucocyte adherence inhibition technique, first described for the study of experimental tumours in mice (Halliday and Miller, 1972; Halliday *et al.*, 1974 b), has recently been applied to the detection of specific cell-mediated immunity in human cancer (Halliday *et al.*, 1974 a; Maluish and Halliday, 1974). Patients with melanoma and carcinoma of the colon and breast were found consistently to have detectable cell-mediated immunity accompanied by serum blocking factors; this reactivity was specific for tumour type, suggesting a useful application in routine diagnosis. The leucocyte adherence inhibition test is simple to perform and requires no complex equipment or cultivation or labelling of tumour cells.

We describe here the application of the test to the diagnosis of malignant hepatoma. The technique gave consistently positive results in confirmed hepatoma and enabled this malignancy to be distinguished from secondary hepatic tumours, non-malignant liver diseases, and other tumours not associated with the liver.

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Patients, Materials, and Methods

The patients studied included 7 with primary hepatic tumours (6 with hepatocellular carcinoma, 1 with hepatoblastoma); 3 with secondary hepatic tumours (1 case each of malignant melanoma, adenocarcinoma of the lung, and squamous cell carcinoma of the cervix uteri); 43 with other diseases of the liver, of whom 33 were cirrhotic (9 alcoholics, 8 with chronic active hepatitis, and 16 with haemochromatosis); and 4 with neoplasms not involving the liver (melanoma and adenocarcinomas of the breast, colon, and stomach).

The technique has already been described (Halliday and Miller, 1972; Halliday *et al.*, 1974 a, b; Maluish and Halliday, 1974). The test is based on the observation that when blood leucocytes from a patient with cancer are mixed with the corresponding specific tumour antigen the tendency of the leucocytes to adhere to glass is inhibited. Serum from such patients interferes with or "blocks" this inhibition.

Leucocytes were separated from heparinized venous blood (15 ml) by settling in glass bottles, removing erythrocytes by osmotic lysis, and adjusting to 2×10^7 cells/ml in Eagle's medium plus fetal calf serum. Serum from the patients was obtained in the conventional way from clotted blood. Tumour extracts (antigens) were prepared by homogenizing tumour tissue in isotonic phosphate-buffered saline and centrifuging at 20,000 g. Separate extracts were made from hepatoma tissue and from histologically normal liver tissue obtained at necropsy from the same patient (case 4). The supernatant fluids, containing 4-8 mg of total protein/ml, were stored at -20°C .

For the determination of adherence duplicate mixtures of leucocytes (10^6 in 0.05 ml), antigen where required (diluted 1/5; 0.05 ml), serum (from patients or normal controls; 0.05 ml), and Eagle's medium (to a total volume of 0.2 ml) were incubated for 30 minutes at 37°C then introduced into haemocytometers. After further incubation for 60 minutes in humid chambers (large Petri dishes are convenient) cells were counted in a pre-determined pattern of squares (16 squares per haemocytometer). The cover slips were floated off in Hanks's solution (contained in a large Petri dish), the slides rinsed, and the remaining adherent cells counted in the same squares. All counting was performed without knowledge of the contents of the mixtures, which were coded by another person. Finally, the mean percentage of adherent cells and the standard error (S.E.) of the mean was calculated for each mixture and the significance of the comparisons determined by Student's *t* test. Where a positive result (+) is reported the significance of the difference between test and control values was highly significant ($P < 0.001$).

Sera were also tested for α -fetoprotein by immunodiffusion and for antigen and antibody to hepatitis B (HBsAg and HBsAb) by solid-phase radioimmunoassay (Peterson *et al.*, 1973). Liver biopsies were performed by the Menghini technique, and the criteria used for the diagnosis of chronic active hepatitis and idiopathic haemochromatosis were those of Cooksley *et al.* (1972) and Powell (1970) respectively.

Results

Clinical and laboratory data on the patients with primary liver

tumours (cases 1-7) and on two with secondary liver tumours (cases 8 and 9) are set out in table I. Serological tests were negative for α -fetoprotein, HBsAg, and HBsAb in all but five patients with non-malignant liver disease; of the remainder two patients with chronic active hepatitis were positive for HBsAg and three (two with haemochromatosis and one with chronic active hepatitis) were positive for HBsAb.

Results of preliminary tests for specific and non-specific activity of the antigen extracts are shown in table II. Neither the normal liver extract nor hepatoma extract affected the adherence of normal leucocytes or leucocytes from a patient with melanoma. The hepatoma extract but not the normal control extract inhibited adherence of leucocytes from a patient with hepatoma. These are representative results which were repeated many times. The cell-mediated immunity shown by this test was examined further for specificity by testing the reaction of leucocytes from patients with hepatoma against extracts of other tumours and normal tissues. The results (table III) showed that only hepatoma tissue yielded an antigenically active extract though the other tumour extracts were specifically active with leucocytes from appropriate patients (Halliday *et al.*, 1974 a; Maluish and Halliday, 1974).

TABLE II—Effect of Tissue Extracts on Leucocyte Adherence

Leucocytes	Extract (Antigen)	% Adherence (Mean $\pm$ S.E.)	Result	P
Normal	None	68.1 $\pm$ 2.2	Normal adherence	>0.2
	Normal liver	65.0 $\pm$ 1.6	No inhibition	
Normal	None	74.6 $\pm$ 2.0	Normal adherence	>0.6
	Hepatoma	73.0 $\pm$ 2.3	No inhibition	
Melanoma	None	71.7 $\pm$ 2.4	Normal adherence	>0.1
	Hepatoma	67.5 $\pm$ 2.3	No inhibition	
Hepatoma	Normal liver	75.8 $\pm$ 1.5	Normal adherence	<0.001
	Hepatoma	49.3 $\pm$ 1.7	Specific inhibition (35%)	

TABLE III—Reaction of Leucocytes from Hepatoma Patients with Various Extracts

Extract	%Adherence of Leucocytes (Mean $\pm$ S.E.)			
	Case 1		Case 6	
	20/10/1973	22/10/1973	4/10/1973	15/10/1973
None	62.3 $\pm$ 1.9		70.6 $\pm$ 3.1	
Hepatoma	42.0 $\pm$ 2.0	46.4 $\pm$ 2.4	42.5 $\pm$ 2.3	52.2 $\pm$ 2.4
Normal liver		69.9 $\pm$ 2.1	72.7 $\pm$ 2.0	75.8 $\pm$ 1.5
Fetal liver (20 week)		68.7 $\pm$ 2.6		
Carcinoma of stomach		68.2 $\pm$ 1.8		75.6 $\pm$ 1.7
Carcinoma of colon		73.8 $\pm$ 1.9		81.0 $\pm$ 1.1
Carcinoma of lung (secondary deposit in liver)		68.1 $\pm$ 2.4		77.8 $\pm$ 1.2

A further distinctive feature of hepatoma was shown when patients' sera were included in the reaction mixtures (table IV). These sera were observed to block leucocyte adherence inhibition, not only when a patient's own serum was used with his cells but also when the serum and cells originated from different patients with hepatoma. Serum blocking was thus related to tumour type, as was cellular reactivity. Sera from patients with

TABLE I—Characteristics of Patients with Hepatic Tumours

Case No.	Age (Years)	Type of Tumour	Associated Liver Disease	Hepatic Scan	Confirmation of Diagnosis	α -Feto-protein	HBsAg	HBsAb
1	70	Hepatoma	Haemochromatosis with cirrhosis	Space-occupying lesion	Liver biopsy	—	—	—
2	58	Hepatoma	None	Space-occupying lesion	Liver biopsy	—	—	—
3	73	Hepatoma	Haemochromatosis with cirrhosis	—	Necropsy	—	—	—
4	75	Hepatoma	Haemochromatosis with cirrhosis	Space-occupying lesion	Necropsy	—	—	—
5	19	Hepatoma	Chronic active hepatitis and cirrhosis	Space-occupying lesion	Necropsy	+	—	—
6	52	Hepatoma	Alcoholic cirrhosis	Space-occupying lesion	*	+	—	—
7	10	Hepatoblastoma	None	Space-occupying lesion	Liver biopsy	+	—	—
8	52	Secondary (cervix)	None	Space-occupying lesion	—	—	—	—
9	60	Secondary (melanoma)	None	—	—	—	—	—

*Biopsy showed only cirrhosis. Serial scans indicated rapidly enlarging space-occupying lesion.

other types of tumours, though blocking with the corresponding antigen, were unreactive in the hepatoma system (table IV). Conversely serum from patients with hepatoma did not block the interaction of leucocytes and antigens derived from other types of tumours. All of six patients with hepatoma were shown consistently to have specific blocking factor in their serum, some being tested on several occasions over a period of two months (table V). Four of these had specific leucocyte activity consistently on repeated testing; leucocytes from the other two were unavailable.

TABLE IV—Effect of Different Sera on Leucocyte Adherence Inhibition

Source of Leucocytes	Antigen	Serum	% Adherence (Mean ± S.E.)	Result	P
Case 1	Normal liver	Normal	69.9 ± 2.1	Normal adherence	<0.001
	Hepatoma	Normal	46.4 ± 2.4	Leucocytes active	
	Hepatoma	Case 1	71.3 ± 2.4	Serum blocking	
Case 6	Normal liver	Normal	75.9 ± 1.5	Normal adherence	<0.001
	Hepatoma	Normal	52.2 ± 2.4	Leucocytes active	
	Hepatoma	Case 6	75.6 ± 2.4	Serum blocking	
Case 1	Hepatoma	Case 4	72.9 ± 1.8	Serum blocking	<0.001
	Hepatoma	Normal	67.3 ± 2.5	Normal adherence	
	Hepatoma	Normal	45.1 ± 2.4	Leucocytes active	
Case 1	Hepatoma	Case 9	48.6 ± 2.2	Serum not blocking	<0.001
	Hepatoma	Normal	68.9 ± 1.9	Normal adherence	
	Hepatoma	Normal	39.9 ± 2.9	Leucocytes active	
Case 1	Hepatoma	Case 10*	37.0 ± 1.8	Serum not blocking	<0.001
	Hepatoma	Case 10*	37.0 ± 1.8	Serum not blocking	

*Case of carcinoma stomach.

TABLE V—Reactions of Known Hepatoma Patients to Leucocyte Adherence Inhibition Tests

Case No.	Leucocyte Activity	Serum Blocking
1	+ (9 Times†)	+ (6 Times)
2	+	(2 Times)
3	Not tested	+
4	Not tested	(2 Times)
5*	+	+
6*	+ (6 Times)	+ (6 Times)

*In these patients a positive leucocyte adherence inhibition test result was the first definite indication of malignancy.
†Percentage inhibition of leucocyte adherence on each occasion (with leucocytes obtained at intervals during October to November 1973): 33.9, 50.7, 33.0, 35.0, 32.8, 53.5, 35.5, 39.7, 20.5.

Stored sera from two patients (cases 1 and 4) who had been treated for haemochromatosis for several years were also tested (using fresh reactive leucocytes) (table VI). Serum obtained in 1969 from case 1 was unreactive but samples taken in 1971 and 1972 were blocking. Serum from case 4 taken in 1970 and 1971 when hepatoma was not suspected was found to be blocking.

In contrast to the uniformly positive results obtained with patients suffering from hepatoma tests done in other situations gave negative results except in four cases of haemochromatosis. These patients showed blocking factor in their serum on repeated testing. Leucocytes were available from only one of these patients with blocking serum, and they were found to be specifically active. All cases of cirrhosis, chronic active hepatitis, and secondary hepatic tumours were negative for serum blocking; the single patient with hepatoblastoma had no hepatoma-related activity in either leucocytes or serum.

TABLE VI—Progressive Changes in Immunological Status in Relation to Clinical Appearance of Tumour

	1969	1970	1971	1972	1973
Case 1	..	..	..	..	H
Case 4	..	..	..	..	H

— Serum not blocking. + Serum blocking. H = Clinical diagnosis of malignant hepatoma.
*Detailed case report appeared at this time indicating apparent complete reversal of hepatic "cirrhosis" (Powell and Kerr, 1970).

Discussion

Our results show that as with other human tumours (Halliday *et al.*, 1974 a; Maluish *et al.*, 1974) leucocyte adherence inhibi-

tion provides a rapid, sensitive, and specific test of value in diagnosis. All patients with proved malignant hepatoma gave positive reactions, and in two (cases 5 and 6) this was the earliest detected manifestation of tumour, resulting in subsequent clinical investigation and diagnosis. In contrast patients with other tumours in the liver (primary or secondary) did not react with the hepatoma antigen and could easily be distinguished.

Some characteristics of the test which constitute technical improvements on other techniques in tumour immunology were noted previously (Halliday and Miller, 1972). In connexion with the present studies we stress rapidity and reliability. A single operator can complete a series of tests—for example, all of those shown in table II or the examination of 8 to 10 sera for blocking against known reactive leucocytes—in one working day; media and antigens are conveniently prepared in advance and stored. Reliability is shown in several ways—the reproducibility of results from day to day (as with blocking activity of the same serum sample), the consistency of any given patient's reactions at different times (leucocytes and serum from cases 1 and 6 were always reactive on many separate occasions), and the constant association between positive reactivity and tumour status, as shown in table V.

The nature of the blocking factor in human hepatoma serum has not been studied in detail but there is much information on experimental tumours of rats. Blocking activity related to such tumours could be produced by mixing purified hepatoma antigen with specific antitumour antibody (Baldwin *et al.*, 1972), thus supporting an earlier suggestion (Sjögren *et al.*, 1971, 1972) that blocking factors in certain other tumour systems are antigen-antibody complexes. The proposal that blocking activity may be caused by tumour antigen alone (Currie and Basham, 1972) is not compatible with our findings, since antigen causes inhibition of adherence in these tests, whereas blocking factor has the opposite effect. It is not known whether the cells, antigens, and host responses detected by leucocyte adherence inhibition are exactly the same as those studied by lymphocyte cytotoxicity; superficially they seem to be very similar in terms of specificity and association with tumour growth. It seems unlikely that the hepatoma-associated antigen detected by the test is α -fetoprotein. This antigen was found in only three of the seven patients with primary liver tumours. The active antigen, though found in hepatoma extract, could not be detected (table III) in an extract of fetal liver which presumably contained α -fetoprotein.

Of particular interest was the relation of leucocyte adherence inhibition reactivity to early tumour development. As mentioned above, in cases 5 and 6 a positive test result preceded other clinical and laboratory evidence of hepatoma (table V), and serum from cases 1 and 4 (table VI) was blocking two and three years respectively before the clinical appearance of their tumours. In a further four patients with primary idiopathic haemochromatosis, which is known to predispose to malignant hepatoma, blocking factor has been detected in the serum (with reactive leucocytes also in one case) but as yet there is no other clinical or laboratory evidence of malignancy. It is suggested that cell-mediated immunity and serum blocking may be the earliest detectable manifestation of malignant hepatoma in these patients.

Note added in Proof.—The patient (Case 6) whose diagnosis was unconfirmed has recently died; necropsy examination showed malignant hepatoma.

This work was supported by the National Health and Medical Research Council of Australia, the Queensland Cancer Fund, and the University of Queensland Cancer Research Fund. We thank medical and surgical colleagues for their co-operation, Mr. C. Jacobi and Mr. K. L. Gera for serological studies, and Miss Suzanne Roy-Smith for technical help.

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Energy Provision, Tissue Utilization, and Weight Loss in Prolonged Starvation

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British Medical Journal, 1974, 2, 352-356

Summary

Daily weight loss measurements in 76 fasting, obese patients (58 females, 18 males) have shown a characteristic pattern of rapid loss initially (up to day 14) followed by a slower but uniform reduction in weight. There were significant sex differences. Measurements of tissue breakdown showed that the initial rapid weight loss was due to the continuing utilization by the nervous system of glucose derived from lean tissue. This requirement fell noticeably from the second week of starvation. Irreversible fluid elimination also contributed to the initial phase of rapid weight loss. Fat remained the primary source of energy throughout starvation and in established fasting (more than 14 days) contributed 96% of that requirement.

Introduction

Many obese patients can undergo prolonged starvation and lose large amounts of weight (Drenick *et al.*, 1964; Thomson *et al.*, 1966; Runcie and Thomson, 1970). Under these conditions the rate of weight loss is greatest initially and thereafter falls off (Rapoport *et al.*, 1965). It has been suggested that weight loss in starvation results from the breakdown of lean rather than adipose tissue (Benoit *et al.*, 1965). We have reviewed the weight loss responses in 76 fasting, obese patients (58 females and 18 males) throughout extended starvation to define the normal response. Since there are no large carbohydrate

reserves in the body metabolic energy needs can be met only by the catabolism of fat in adipose tissue or protein in lean body mass or of some combination of these two—the metabolic “fuel mix.” In this study breakdown of lean tissue was measured continuously throughout fasting in a number of patients as either total protein degradation (urinary nitrogen excretion) or urinary potassium loss to provide a continuous measure of the varying proportions of lean tissue and fat metabolized. These values were also used to calculate the mean energy production of fasting, obese subjects.

Patients and Methods

The patients' obesity was either massive or refractory to treatment or they suffered from serious intercurrent disease aggravated by obesity. Before fasting all patients received a normal ward diet. The therapeutic regimen consisted simply of no food. Water-soluble vitamins (Multivite tablets) were the only supplements allowed. Water was not restricted. Patients adopted their own level of physical activity. No attempt was made to measure energy utilization direct, primarily because of the long starvation periods employed. Patients were weighed daily after rising and emptying the bladder. The urine of 35 patients was collected continuously throughout fasting and an aliquot of each 24-hour specimen was analysed for its potassium content by a standard flame photometer technique. In 28 patients (18 females and 10 males) urinary nitrogen excretion was measured by a macroKjeldahl method incorporating a multidistillation apparatus.

In seven females changes in whole-body potassium throughout starvation were measured by a scanning type of whole-body monitor. The machine is housed in a custom-built steel room. It consists of a ring of six sodium iodide detectors, three above and three below the patient. Each detector is 15 cm in diameter and 10 cm in depth. An estimation takes 44 minutes. Calibration data, principally to take account of the differences in absorption of radiation by subjects of varying size and body thickness, were obtained by extensive studies with ⁴⁰K-filled phantoms. Water-filled bags were used to simulate varying thicknesses of adipose tissue. The results were similar to the calibration data of Cohn and Dombrowski (1970) for a monitor of comparable detector geometry and are shown in fig. 1. The standard deviation for each estimate of total body potassium, including statistical and calibration errors, was 4%.

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