No failure of erythropoiesis or erythrocyte maturation was observed.

It is claimed that death is due to anaemia consequent upon thrombocytopenia and haemorrhage.

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SCHULTZ-DALE TEST FOR DETECTION OF SPECIFIC ANTIGEN IN SERA OF PATIENTS WITH CARCINOMA

BY

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The search for a reliable and easily performed serological test for carcinoma has interested many investigators for over 60 years, and the number of publications on the subject now runs into several thousands. Homburger (1950) reviewed over 60 tests described in the last 35 years, and none conformed to the strict criteria laid down by Dunn and Greenhouse (1950) of 90% accuracy in cases of cancer, 95% accuracy for patients with non-cancerous disease, careful selection of cases and controls for testing, need for reproducibility in different hands, knowledge of the extraneous factors affecting the cases, and sufficient numbers tested to be statistically significant. Many people feel that it is unscientific to search for a serological test for cancer on account of the diversity of causes and types, and there is no doubt that finding a suitable test is made more difficult by lack of a full knowledge of the cause of human cancer. With other diseases the discovery of the aetiological agent has usually been followed by a simple serological diagnostic test—for example, Widal test, etc.—and it is reasonable to hope that cancer might similarly produce some constant and specific change in the serum which would prove of diagnostic value. Greenstein (1954) has said, "Tumours tend chemically to resemble each other more than they do normal tissue or than normal tissues resemble each other."

A test for carcinoma described by Makari seemed to fulfil the criteria of Dunn and Greenhouse with the exception of information about reproducibility by other workers; furthermore, it was based on two assumptions which it seemed reasonable to make (Makari and Huck, 1955). These are as follows.

1. Any carcinoma in the body will release into the circulation a substance specific to carcinoma tissue and not found in normal patients, probably a molecule or part of a molecule making up the substance of the tumour itself. It has been shown that this is indeed so by Mann and Welker (1940, 1943), who stated that the "proteins of carcinoma tissue may appear in the blood stream and be detected by the appropriate antiserum."

2. It is possible to produce a reaction between this unknown substance and antibodies in a guinea-pig which has been injected with carcinoma. As mentioned above, Mann and Welker (1940, 1943) were able to detect an abnormal substance in cancer sera by using antibodies and a precipitin reaction.

Makari's methods consisted in using one of the most sensitive methods for detecting antigen—the Schultz-Dale bath. He tried several antigens from carcinoma tissue for sensitizing guinea-pigs; the most successful of these gave 96.8% positive results in proved carcinoma and only 4.8% false positives in non-cancerous lesions.

It is the purpose of this article to show that with some modification we have been able to confirm and slightly improve upon these results in a much larger series.

Method

The technique of performing the test was substantially the same as that used by Makari: the only material used for sensitizing guinea-pigs was the one Makari found to give the best results. This fraction was obtained by making a 20% aqueous emulsion of any carcinoma, centrifuging at 3,200 r.p.m., equivalent to an R.C.F. of 1.5 kg./g, and then recentrifuging the supernatant at 4,000 r.p.m., equivalent to an R.C.F. of 2.3 kg./g; the deposit was then injected subcutaneously in Freund's adjuvant into virgin guinea-pigs, 200-600 g. in weight. This has the effect of removing to some degree the non-specific normal fibrous tissue, blood, and blood vessels. Many differing types of carcinoma, including metastases, were investigated. Those actually used were: adenocarcinoma of breast; adeno-, squamous-, and oat-cell carcinoma of lung; adenocarcinoma of rectum, colon, stomach, and prostate; hypernephroma; chorion-epithelioma; and glioblastoma.

One month later the guinea-pigs were killed and the uterine horns suspended in a Schultz-Dale bath. The horns were desensitized to normal elements in plasma by the addition of 1.5 ml. of normal plasma, until no further contractions were recorded, washing the uterus and allowing it to relax fully between each contraction. 1.5 ml. of test serum was then added to the immune horn and a normal horn. If the patient had a carcinoma the immune horn should have contracted, but not the control one. The criterion accepted by Makari for a positive test was one which gave a contraction which was at least one-fifth the size of that produced by 10 µg. of histamine phosphate. In this work this criterion was found to be adequate, for, though the test was not an all-or-nothing phenomenon, the positive sera generally produced a big contraction, and had the standard been one-third or one-tenth it would have made little difference to the results.

Two modifications of Makari's technique were made. (1) It was found that the uteri of guinea-pigs lost their contractibility in the winter. This was restored by five injections of 0.2 mg. of oestradiol benzoate subcutaneously at three-day intervals before sacrifice. (2) It became obvious early in the work that a large proportion, 30-40% of guinea-pigs, did not become adequately sensitized, sometimes to the carcinoma element only, sometimes to the normal elements as well, and so when a negative result was obtained with a test serum, serum from a patient with proved carcinoma which had previously given a positive test was added to the uterus. If there was still no contraction the uterus was regarded as inadequately sensitized and the result disregarded.

Results

Of the 301 patients with carcinoma tested, 291 were positive (Table I), an accuracy of 96.7%. The 10 false negatives were: early squamous carcinoma of tongue, carcinoma of thyroid, astrocytoma, post-cricoid squamous carcinoma, malignant melanoma with secondaries in brain, malignant carcinoid?, adenocarcinoma of pancreas with secondaries

TABLE I.—Results in 291 Cases of Carcinoma

Carcinoma	Primary Only	Meta-stasis	Recur-re	Total
Adenocarcinoma of breast ..	22	11	9	42
Cerebral tumours ..				37
Carcinoma of lung (all histological types) ..	15	20	1	36
Adenocarcinoma of stomach ..	13	10		23
" prostate ..	4	16		20
" colon ..	13	5	1	19
" rectum ..	10	5		15
Squamous carcinoma of skin ..	10	1		11
Carcinoma of cervix ..		3		9
Transitional cell carcinoma of bladder ..	6			9
Carcinoma of larynx ..	5	3		8
Squamous carcinoma of tongue ..	8			8
Adenocarcinoma of ovary ..	5	2		7
Squamous carcinoma of oesophagus ..	4	3		7
" vulva ..	6			6
Adenocarcinoma of pancreas ..	4	2		6
Carcinoma of thyroid ..	5			5
" pharynx ..	4			4
Basal-cell epithelioma ..	4			4
Malignant melanoma ..	2	2		4
Adenocarcinoma of body of uterus ..	4			4
Carcinoma of lip ..	3			3
Seminoma testis ..	1			1
Chorionepithelioma of testis ..	1			1
Hypernephroma ..	1			1
Squamous carcinoma of anus ..	1			1

TABLE II

Patient's Condition:	No. Tested
Normal ..	20
Sarcoma (leiomyosarcoma 5, lymphosarcoma 4, reticulum-cell sarcoma 3, fibrosarcoma 2, liposarcoma 1, osteogenic sarcoma 1) ..	16
Nephrosis ..	13
Cerebral thrombosis ..	12
Molluscum sebaceum ..	10
Recent coronary infarction ..	10
Leukaemia ..	9
Congestive heart failure ..	9
Normal pregnancy ..	8
Peptic ulcer ..	8
Hypertension ..	6
Active pulmonary tuberculosis ..	6
Syphilis ..	5
Disseminated sclerosis ..	5
Pneumonia ..	5
Jaundice ..	4
Multiple myeloma ..	3
Rheumatic heart disease (without congestive failure) ..	3
Increased intracranial tension (no malignant cause found) ..	3
Psoriasis ..	3
Pituitary adenoma ..	3
Neurofibroma ..	2
Infarct lung ..	2
Cystic mastopathy ..	2
Abdominal adhesions ..	2
Fibromyomata uterus ..	2
Diabetes ..	2

One each of the following was tested—Lupus erythematosus, brachial neuritis, brain abscess, chronic cervicitis, cystitis, alcoholic patient, after repair operation, mesothelioma pleura, subdural haematoma, exfoliative dermatitis, metropathia, oesophagitis, sebaceous cyst, Simmonds's disease, polycystic kidneys and hypertension, recurrent desmoid tumour of abdominal wall, simple ovarian cyst (serous), basal-cell papilloma, leuco-erythroblastic blood dyscrasia, muscular dystrophy, Hodgkin's disease, leucoplakia of vulva, non-specific granuloma dermis, malaria, teratoma of ovary, tabes, migraine headache.

in liver, primary squamous carcinoma of larynx, adenocarcinoma of breast with secondaries in axillary glands, secondary adenocarcinoma (unknown primary).

Of the 207 patients without carcinoma tested, 200 were negative (Table II), an accuracy of 96.7%. The seven false-positive results were: simple gastric ulcer, chronic granulomatous lesion of breast, infarct of brain, and four cases of nephrosis.

Ten carcinomas were tested before and three weeks after removal: 6 were adenocarcinomas of breast, 2 were adenocarcinomas of rectum, and 2 were meningiomas. These cases were chosen because so far as could be ascertained all the growth had been removed and there were no metastases, and

four months later they were still free of recurrence. Of the eight carcinomas of breast and rectum, five had become negative and three were still positive. The two meningiomas were still positive, but on testing them two months later they had become negative, as had the other three positive cases.

Seven carcinomas were successfully removed but not tested prior to removal. The length of time since removal was:

Squamous carcinoma of oesophagus ..	7 years
Adenocarcinoma of uterus ..	6 "
" colon ..	6 months
Squamous carcinoma of lung ..	3 "
" larynx ..	6 weeks
Adenocarcinoma of breast ..	3 "
Meningioma ..	3 "

The only extraneous factor which appeared to influence the result of a test was haemolysis in the serum, which tended to produce a false-positive result; other factors such as keeping several days at room temperature or several months at -40°C . had no effect on the result.

Makari stated that if he placed a normal uterus in serum containing antibodies to carcinoma then the muscle adsorbed the antibodies and was as suitable for testing as the uterus from the primarily sensitized guinea-pig. This was not confirmed, and on studying Dale's (1913) original article it was seen that this was also what he found, but for some reason or other he was able to sensitize passively in this way muscle which had previously been actively sensitized and subsequently desensitized.

Sarcomas.—When it was noted that a positive test was not elicited with sarcomatous sera it was important to discover if sera from patients with sarcoma would produce a positive reaction when tested in guinea-pigs sensitized to sarcoma; so a reticulum-cell sarcoma, a lymphosarcoma, a fibrosarcoma, and a leiomyosarcoma were extracted in the same way as the carcinomas, injected into guinea-pigs, and tested in the Schultz-Dale bath. It was not possible to produce one positive result from 12 sera from different patients with sarcoma and five patients with leukaemia. This is peculiar in view of the specificity of the test for carcinoma and there is no obvious explanation for it.

Discussion

Quantitative Aspects.—The test has not been found to be a useful quantitative measurement of the amount of tumour present in the body, for it was found that small carcinomas often produced as large a contraction as serum from a patient with a large bulk of tumour. The test therefore is of no value in assessing the amount of carcinoma present in a patient, and no attempt has been made to carry it further than to elicit a positive or negative result. This is understandable, as the size of contraction is dependent on two factors: one is the amount of abnormal substance released from the tumour, which will depend on many different facts as well as size—for example, the degree of differentiation, vascularity, necrosis, and the organ in which the tumour is growing. The other more important factor is the amount of antibody present in the uterus; this is very variable.

It is important to know how small a tumour the test will reasonably detect. This was readily seen in the studies of small visible tumours—for example, skin, lip, and tongue. In this group the test was found to be very accurate down to tumours of $\frac{1}{2}$ in. (1.3 cm.) in diameter. When they were smaller accuracy decreased, and very small squamous carcinomas and basal-cell epitheliomas were negative. This means that the test is valueless in detecting small areas of malignant change—for example, in a polyp or leukoplakia; neither can a good prognosis be given merely because the test becomes negative post-operatively. It is also of importance to realize that a negative result may not become established till some weeks after operation.

False Negatives.—The squamous carcinoma of the tongue and larynx were both small tumours with no invasion or

metastasis, and they both had a strikingly large number of inflammatory cells, particularly plasma cells round the tumour. It may be that these plasma cells were producing antibodies to the tumour and were neutralizing any abnormal substance released from it. The carcinoma of the thyroid was a very well differentiated tumour and might well have been called a benign adenoma but for an area of venous invasion. The astrocytoma was also a well-differentiated and slowly growing tumour, and it may be that these latter two tumours were not dedifferentiated enough to release a substance specific to carcinoma and were chemically more akin to benign tumours, but it should be noted that many well-differentiated carcinomas did give positive tests. The case of carcinoid tumour is the only tumour which was not histologically proved, but it has been accepted as such on the serological levels of 5-hydroxy-tryptamine and the clinical findings. With this in mind it is possible that there might be a reasonable explanation for five of the false negatives.

False Positives.—The fact which stands out is the high percentage of false positives in patients with nephrosis. There are many explanations of this finding, including the possibility of antidiuretic hormone causing a non-specific contraction, but it should also do so in the non-sensitized horn; or there may be a substance in the serum of nephrosis similar to that found in carcinoma serum which is responsible for the positive reaction. In the course of this work chromatographic investigations of urine of patients with nephrosis and carcinoma (Burrows and Neill, 1958) suggest that this may be so. So far there is no satisfactory explanation.

Probably no serological test for cancer will ever be of use for screening large normal populations, on account of the large number of normal people who would have positive tests and have to be investigated for cancer, and this test is no exception. Where it has been found of great value is in the study of patients in whom a question of carcinoma is raised in diagnosis; but the most valuable aspect of the test has been in assessing patients who have had a carcinoma removed some time previously and have returned complaining of symptoms which could mean recurrence of the original growth. All patients in this group gave a positive result.

Summary

A test described by Makari and Huck for serological diagnosis of carcinoma has been investigated. There was 96.7% accurate diagnosis in 207 patients with non-carcinomatous disease and 96.7% accurate diagnosis in 301 cases of histologically proved carcinomas.

The use and limitations of the test are discussed.

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INVESTIGATION OF THE IMMUNOLOGICALLY ACTIVE CONSTITUENT OF SERUM OF PATIENTS WITH CARCINOMA

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The demonstration in the serum of patients with carcinoma of the presence of a substance capable of initiating contractions in the immune guinea-pig uterus suggested a study of such sera to elucidate its nature. In the absence of any information on its structure beyond its immunological similarity to some component of the carcinoma itself an attempt was made to separate suitable sera on the basis of physical or chemical properties and to test each of the separated fractions for specific activity in the Schultz-Dale bath. Although a number of fractionation techniques were used it was found that in most cases efficient separation was achieved only at the expense of partial denaturation of the protein constituents of the serum. Paper electrophoresis did, however, achieve reasonably good separation of the serum proteins without degradation, and apparatus was set up to achieve separation on a preparative scale by the technique of continuous electrophoresis.

By this method the serum to be fractionated is applied to the top edge of a hanging sheet of filter paper down which buffered electrolyte flows, and along the vertical edges of which electrodes are applied which enable a potential difference to be applied across the sheet. Thus in travelling down the paper with the flowing electrolyte, the ionized components of the serum migrate towards their appropriate electrodes, at rates which are a function of their electrophoretic mobility. As equilibrium is established, the slow-moving anions form bands across the sheet, and can be collected by arranging tubes along serrations cut in the bottom edge.

Method

The continuous electrophoresis apparatus used was made up after the design of Holdsworth (1955). The flowing electrolyte was a barbitone buffer pH 8.6 made by dissolving 1.84 g. of barbitone and 10.3 g. of sodium barbitone to 1 litre of aqueous solution. The apparatus accommodated paper sheets 17 in. (43 cm.) horizontal by 20½ in. (52 cm.) vertical, and in use a potential difference of 1,000 v. was applied to platinum electrodes in contact with the vertical edges of the wet paper. The serum was diluted 1:1 with buffer and applied, so diluted, at the rate of 0.5–1 ml. per hour by means of an automatic slow-motion drive to the plunger of an all-glass syringe. Adequate separation was achieved in 24–48 hours, during which time the serum flow rate was adjusted to apply a total volume of 10–15 ml. The buffer flowing from the serrated bottom edge of the sheet carried the separated fractions into collecting tubes. At the end of the run the positions of the separated fractions across the sheet were determined by immersing the sheet in a dye-bath of the wool dye lissamine green S.F.; after rinsing in 1% aqueous acetic acid the serum protein components showed as green bands.