SERUM DETECTION OF CARCINOMA

EXPERIENCE WITH THE SCHULTZ-DALE TECHNIQUE

BY

EARLE HACKETT, M.A., M.D., M.C.P.A.

AND

EDITH GARDONYI, M.D.

*The Institute of Medical and Veterinary Science, Adelaide,
South Australia*

Makari (1955, 1958), using the Schultz-Dale technique, has reported the detection of characteristic antigens in 95% of sera from patients with carcinoma, with fewer than 5% false results in non-carcinomatous patients and healthy persons. Burrows (1958) confirms this, but McEwen (1959) does not. Makari (1959) criticizes McEwen's method, and gives some previously unpublished details of his own technique.

Further independent investigation of this cancer test is very necessary. If the reliability claimed by Makari and by Burrows could be reproduced in a few more centres the test would be worth setting up, in spite of its technical difficulties, in all major medical laboratories.

We have attempted the method, and our results are reported here.

Outline of the Method.—A virgin female guinea-pig is immunized with an extract of carcinoma. After an interval the animal is sacrificed and a uterine horn is mounted in a Schultz-Dale bath of warm Tyrode solution. The contractions of the horn are recorded on a kymograph. The muscle is first desensitized with pooled normal human serum which produces an anaphylactic contraction (for the animal will have become immune to normal components in the carcinoma extract), after which a further specific contraction should occur upon challenge with serum from a carcinomatous patient. Skill is acquired slowly, and the test involves so many variable factors that it is at present most unlikely to be performed in exactly the same way in any two laboratories. Two important papers on the details of the Schultz-Dale technique are those by Dale (1913) and Coulson (1953).

Controls.—Makari (1955) stated that "a horn from an unsensitized normal guinea-pig was used as a control" and that "any serum which caused a contraction of the normal horn could not be evaluated." Burrows (1958) also used a normal horn as a control. In a later paper Makari (1958) no longer refers to an unsensitized horn, using instead the uterine horn of a guinea-pig immunized with non-cancerous human tissue. In his comments on McEwen's report Makari (1959) says that the use of a normal unsensitized control horn, upon which McEwen had relied, is a "definite misconception." He adds: "Early in our work we have used this procedure in one experiment on sera from patients with other diseases than cancer to detect a pressor effect. We have since found that, when cancer tissue antigens or sera from cancer patients are added to the unsensitized horn, positive reactions appear in many of these." If the first sentence is correct, then his earlier paper (Makari, 1955) is misleading. However, we believe that his present view is permissible, and we have adopted it. It implies that non-specific pharmacological pressor agents or responses will be revealed without using a normal-horn control, either during the preliminary

desensitization of the sensitized horn with normal serum or by the fact that a contraction which follows challenge with cancer serum should be of the desensitizing type and not repeatable. In the latter case it is necessary to show that the horn is still alive at the end of the experiment, and this can be done by exciting a contraction with histamine.

Tyrode Solution.—An authoritative (*British Pharmacopoeia* 1932) standard Tyrode solution for the guinea-pig uterus is NaCl 9 g./l.; KCl 0.42 g./l.; CaCl₂ 0.24 g./l.; NaHCO₃ 0.5 g./l.; MgCl₂ 0.0025 g./l.; dextrose 0.5 g./l. Dale (1913) reduced "the usual" 0.24 g./l. of CaCl₂ to half strength at 0.12 g./l. (This is the strength McEwen adopts, and is what we use ourselves.) Makari (1955) used CaCl₂ at 0.05 g./l., following Coulson *et al.* (1943), and refers to this (Makari, 1959) as "half strength," when in terms of Dale's original paper it could be described as quarter-strength. Makari (1959) also recommends that MgCl₂ should be increased to 0.1 g./l., and that the Tyrode solution should be adjusted to pH 7.4, and made up freshly every day.

The above points concerning controls and Tyrode solutions are mentioned in detail because many of them have been a source of confusion to us, and perhaps to others.

Materials and Methods

Guinea-pigs.—Young virgin females were used, segregated after weaning. They were taken from the stock bred in this institute in pens of about one hundred animals in a temperature-controlled animal-house. Three to six weeks after immunization they were killed by a blow on the neck and immediately exsanguinated by cutting the throat. The uterine horns were removed quickly and gently without the serous covering being allowed to dry, and placed in warm oxygenated Tyrode solution.

Tyrode Solution.—The *British Pharmacopoeia* 1932 formula for the guinea-pig uterus was made up in glass-distilled water as quoted above, with the CaCl₂ reduced to 0.12 g./l., and adjusted to pH 7.4. It was not made freshly every day, the stock being held at 4° C. in a closed plastic vessel.

Schultz-Dale Bath.—A bath of 10 ml. capacity was used. (Neither Makari nor Burrows gives the bath volume, without which information the final concentration of the challenging serum in solution around the horns cannot be calculated.) The temperature of the bath was controllable to $\pm 0.5^\circ$ C., and was usually set at 36° C. Tyrode solution at the same temperature could be run through at will for the frequent washing of the horn which is required. 95% oxygen with 5% carbon dioxide was steadily bubbled through the bath. The kymograph system had a forward-writing lever and a smoked drum. A tuberculin syringe was used to add measured doses of serum.

Antigen.—This was prepared as Makari's antigen C, sediment III (Makari, 1955), from 100 g. or more of carcinomatous or normal tissue homogenized to a 20% w./v. suspension in normal saline. The homogenate was centrifuged at an R.C.F. of 2.3 kg./g for 15 minutes, the sediment was discarded, and the supernatant recentrifuged at an R.C.F. of 10.3 kg./g for 30 minutes and the sediment retained. When centrifuged in this way the second sediment was always sufficiently fluid to be measured by being drawn into a pipette. It was then resuspended in twice its volume of Difco bacto-adjuvant complete (Freund), and the guinea-pigs were given 0.5 ml.

intramuscularly in the thigh. (Makari does not give details of the doses he injected, but in his 1958 paper he describes the resuspension of the second sediment in the original volume of saline used for the homogenate, and so his preparations may be less concentrated than those we have been using.) The antigen, not mixed with adjuvant, was sometimes stored at -20°C ., but whenever possible (which was usually) it was freshly made before use.

Sera.—Serum, and not plasma, was used for all the tests on patients with carcinoma reported here. 20 ml. of blood was taken, using a glass syringe. After 12 to 24 hours at 4°C . the serum was pipetted off the clot, and stored at -20°C . for up to four weeks. Normal serum was pooled from three donors.

Results

Non-specific Contractions

During a period of eight months, while we gained general experience of the Schultz-Dale technique and Makari's application of it, we investigated non-specific contractions which sometimes occurred when normal or pathological serum was added to uterine horns, both sensitized and normal. McEwen (1959) found these contractions were a major difficulty in the method.

An experiment using 25 normal and 79 sensitized guinea-pigs, and seven normal and five carcinomatous human sera, showed that any serum could produce these contractions if added to the 10-ml. bath in a sufficiently large dose, but that the sensitivity of individual uteri in this respect varied greatly. One contracted with doses of 0.04 ml. of serum, but most of them did so only with quantities of 0.4 ml. or over. In practice, we found that with the particular Tyrode solution we had chosen very few horns would give non-specific contractions if the challenging dose of serum was regulated to 0.5 ml., and so we adopted this rule. We found that when the uteri of 35 sensitized guinea-pigs, some sensitized with carcinoma antigen and some with normal antigen, were satisfactorily desensitized with pooled human serum, not one gave a further contraction when challenged with 0.5 ml. of each of a pair from a panel of 70 normal individual human sera, each guinea-pig being tested with a different pair. We concluded from this that effects produced by individual human serum antigens not represented in the pooled desensitizing serum must be rare.

So far we have been unwilling to reduce the calcium chloride content of our Tyrode solution below 0.12 g./l., for though to do so might abolish non-specific contractility altogether in the horns as we use them, it would also lower the sensitivity of their anaphylactic responses (Coulson, 1953).

Horns from sensitized guinea-pigs gave non-specific contractions with the same average range of doses of serum as normal unsensitized horns. If the injection site became infected (as it did in a number of animals at one stage in our work when an adjuvant-antigen mixture became accidentally contaminated with *B. subtilis*) non-specific contractions became so frequent with minimal doses of serum that the preparations were useless for the Schultz-Dale method. The same happened when some guinea-pigs injured their limbs in the wire mesh of unsuitable cages.

We found no difference between serum and platelet-free heparinized plasma in the tendency to promote non-specific contractions.

Sera from Patients with Carcinoma

Sera from 49 cases of proved carcinoma were tested, using 268 guinea-pigs.

It was decided to rate a serum *positive* only if it (a) produced within 30 seconds a single non-repeatable anaphylactic contraction (after desensitization with normal serum) in both uterine horns (tested separately) of each of three guinea-pigs immunized with carcinoma antigen, and (b) failed to produce contractions in similar tests on both horns of each of two guinea-pigs sensitized with an antigen made from the tissue of a non-cancerous person. Sera were rated *inconclusive* if they gave less than the full number of specific contractions with the six horns in *a* but satisfied *b*, and *negative* if both *a* and *b* gave negative results.

23 guinea-pigs had uterine horns which gave repetitious non-specific contractions during preliminary desensitization with doses of 0.5 ml. of normal pooled human serum. These were discarded. 11 had been sensitized with carcinoma antigens, and 12 with antigens made from apparently normal organs.

Of the 49 sera from carcinomatous patients, 23 were positive (47%), 8 were inconclusive, and 18 were negative, by the criteria given above.

This experiment included an attempt to find whether there was an optimum weight or immunization period for the guinea-pig response. The range of weights of all the animals used was 270 to 510 g., with a mean of 390 g. A greater proportion (not statistically significant) of positively-reacting uterine horns was found in guinea-pigs weighing 420–450 g. No differences were noted between results in guinea-pigs sacrificed at three, four, five, or six weeks after the injection of antigen. Burrows (1958) noticed a seasonal difference in the guinea-pig response, but, so far as we could see, the effects in winter and in summer in Adelaide were the same.

Antigens were made from various carcinomata, some primary and some metastatic, of skin, breast, lung, stomach, and colon. Sera were tested from patients with carcinomata of skin, breast, palate, larynx, lung, stomach, gall-bladder, pancreas, ovary, colon, rectum, and prostate. No significant correlations with site were discernible in our results.

Discussion

Though we cannot substantiate Makari's claims concerning the accuracy of his test in cancer diagnosis, we publish the present account because it offers further confirmation (which we believe was needed) of the two main features of his work. Firstly, it has been shown that there is a difference between the responses of some guinea-pigs immunized with carcinoma antigens and the responses of others immunized with comparable antigens from non-carcinomatous organs. Secondly, these responses are excited by sera from some patients with carcinoma, and not by sera from healthy subjects.

We have not investigated a parallel collection of sera from non-carcinomatous sick people, believing that this is not worth while so long as the proportion of positive results with known carcinoma sera remains as low as 47% in our hands.

We are now using refined extracts of carcinomatous tissues, which we hope will provoke a more reliable immune response in the guinea-pigs, leading to a higher proportion of positive results in the diagnosis of carcinoma, if the substance which triggers the reaction

in the uterine preparation is indeed present in the serum of 95% of patients with cancer, as Makari claims.

Summary

A preliminary attempt to use the Schultz-Dale technique in the diagnosis of cancer has been successful in 23 cases of carcinoma, and unsuccessful in 26.

The claims of Makari and of Burrows that the test has an accuracy of over 95% have not been substantiated. On the other hand, the results can be taken to confirm the immunological basis of the method.

We are grateful to the consulting physicians and surgeons of the Royal Adelaide Hospital and of the Queen Elizabeth Hospital, Adelaide, who suggested that we should test serum from their cases; to Professor R. F. Whelan, of the University of Adelaide Department of Physiology, who gave us much assistance with the setting up of the Schultz-Dale baths and kymograph systems; and to Dr. J. Orde Poynton, Director of this Institute, for helpful interest and advice. This work was assisted by a grant from the National Health and Medical Research Council of Australia.

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THE SERUM LEUCINE AMINOPEPTIDASE TEST

AN APPRAISAL OF ITS VALUE IN DIAGNOSIS OF CARCINOMA OF PANCREAS

BY

J. HARKNESS, M.D., B.Sc.

B. W. ROPER, M.B., Ch.B.

*South Somerset Clinical Area Laboratory, Musgrove Park
Hospital, Taunton*

J. A. DURANT, B.Sc.

AND

H. MILLER, M.D., M.R.C.P.

*Portsmouth and I.O.W. Area Pathological Service, Royal
Portsmouth Hospital*

Despite the multiplicity of laboratory tests of liver function the differential diagnosis of jaundice often presents a vexing problem. This is particularly true when carcinoma of the pancreas is considered, and clinicians would welcome any test which could confirm or exclude such a diagnosis prior to laparotomy.

From an annotation in the *British Medical Journal* (1958) it seemed that such a test was now available in the leucine aminopeptidase estimations as described by Rutenburg, Goldberg, and Pineda (1958). These writers claimed that it was possible to distinguish between patients with carcinoma of the pancreas and patients with other conditions which produce jaundice, by the measurement of the leucine aminopeptidase in serum and urine.

Their results could be summarized as follows:
(a) High serum and high urine values were obtained in all their patients with carcinoma of the pancreas (18 of

head; 2 of tail). (b) Other causes of obstructive jaundice gave high serum values, but not nearly so high as in carcinoma of the pancreas; the urine values sometimes showed a moderate rise. (c) There was a moderate rise, more so in serum than in urine, in other hepatic conditions—for example, cirrhosis, hepatitis, and cholecystitis. (d) Where hepatic metastases were present from primary growths outside the hepato-biliary tract, moderately raised serum levels were found.

This paper is an account of the attempt to confirm the diagnostic value of this test in a large series of patients (including 15 with carcinoma of the pancreas), most of whom suffered from diseases of the liver and bile-ducts.

Method

Leucine aminopeptidase is an enzyme which splits the peptide link between leucine and other amino-acids. It is not absolutely specific, as it can also act on any N-terminal amino-acid with an aliphatic side-chain. In man, it is found in the liver, kidney, spleen, skeletal muscle, alimentary tract mucosa and contents, brain, testis, pancreas, blood, and urine. Its function in the blood and urine is not known.

Each step in the method devised by Goldberg and Rutenburg (1958) was critically examined and verified; their method was finally adopted exactly. The principle of the method depends upon the enzymatic hydrolysis at 37° C. of a buffered substrate of L-leucyl- β -naphthylamine hydrochloride; the liberated β -naphthylamine is converted, by a modification of the Bratton and Marshall (1939) reaction, to a stable azo-dye which can then be estimated colorimetrically against a standard calibration curve prepared from β -naphthylamine hydrochloride. The test is easy to perform and needs no apparatus that will not be found in a routine clinical laboratory. The reagents are now readily available in this country. Serum can be stored at 4° C. for up to a month before analysis.

Goldberg and Rutenburg originally defined the serum activity arbitrarily in terms of the readings on a Klett colorimeter, and the urine activity in terms of the amount of β -naphthylamine liberated under the conditions of the test. As we intended to include renal clearance studies we decided to define our serum activity more specifically and in the same manner as for urine (a unit is the number of micrograms of β -naphthylamine liberated by 1 ml. of a 2% dilution of the serum). In practice, our unit is exactly 12 times as large as that of Goldberg and Rutenburg. These authors later redefined their unit for serum also in terms of β -naphthylamine liberated, and this new unit is also exactly one-twelfth of our unit (Goldberg, Pineda, and Rutenburg, 1959).

A few tests were carried out at the request of our clinical colleagues, but most of the results were obtained from the examination of jaundiced serum received for other investigations and of serum in which the clinical data of the request form indicated the possibility of a condition in which the leucine aminopeptidase might be changed. Fuller clinical information was obtained later from the case-notes or from personal meetings with the clinicians in charge. We discuss only those results where a firm diagnosis could be reached on clinical grounds, together with other laboratory investigations; all cases of carcinoma of the pancreas or secondary malignant deposits in the liver had had a laparotomy or necropsy.