Eur J Cancer Clin Oncol, Vol. 18, No. 7, pp. 651–659, 1982.
Printed in Great Britain.

0277-5379/82/070651-09$03.00/0

A Clinical Assessment of Fluorescence Polarisation Changes in Lymphocytes Stimulated by Phytohaemagglutinin (PHA) in Malignant and Benign Diseases

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Abstract—*The modified double-zone SCM technique, developed in these laboratories as an* in vitro *test for cancer, is based on the differential response to PHA of lymphocytes harvested from two regions of a Ficoll–Triosil gradient. Lymphocyte responses are measured by changes in intracellular fluorescein fluorescence polarisation. We report its continued clinical evaluation in patients hospitalised with malignant and non-malignant diseases, including a blind trial of 78 patients with disorders of the gastro-intestinal tract, breast and lung. Overall 'false' negative and 'false' positive rates from 336 blood samples were 1.8 and 3.3% respectively.*

INTRODUCTION

CERCEK *et al.* [1] reported that the differential response of lymphocytes to phytohaemagglutinin (PHA) and cancer basic protein (CaBP), measured by a fluorescence polarisation technique (SCM), could be used as a basis for an *in vitro* blood test for the detection of malignant disease. Various workers [2–9], some using modified SCM techniques, have confirmed the discrimination between cancer and non-cancer. Two groups [10, 11] have reported failure to reproduce the Cercek results, and possible reasons have been suggested to account for this failure [12–14].

Using a modified double-zone SCM technique in which cells are harvested from two regions or zones of a Ficoll–Triosil gradient, Pritchard *et al.* [15] showed that it is possible on the basis of PHA response alone to distinguish accurately between patients with histologically proven malignancies and those with non-malignant diseases. The present paper describes our experience in the continued clinical evaluation of the double-zone SCM technique, and reports the results of a 'blind' trial to assess the accuracy of the technique in the detection of cancer of the gastro-intestinal (GI) tract, breast and lung. The modified SCM test is now in routine clinical use in this centre.

Accepted 22 January 1982.

MATERIALS AND METHODS

Venous blood samples (20 ml) are taken and transferred into 'Vacutainer' tubes (No. 3208KA) containing 357 I.U. sodium heparin, and processed to yield two fractions of 'SCM responding' lymphocytes. The techniques for handling the samples and for separating the lymphocytes in a modified Ficoll–Triosil gradient of density 1.081 g/ml at 19°C have been described already [3], but several small modifications were introduced during the work reported here: only one layer or band of separated lymphocytes is usually visible in the gradient after centrifuging. A previous account [15] inferred that two 'bands' are commonly seen, so we now prefer the less descriptive term 'zones'. To remove Zone 1 cells, a Pasteur pipette

is held 1 mm above the visible interface and with a single circular motion remove approximately 1.5 ml of the liquid immediately above the interface, which should include the upper part of the (top) visible layer. Zone 2 cells are removed with a similar action by placing the pipette at the interface and removing the rest of the visible layer, together with the liquid directly beneath the interface, to a similar volume. This should include the lower layer when it is visible. An investigation of the relative numbers of cells recovered from different levels along the gradient did not show any significant difference between cancer and non-cancer subjects [16]. After washing, the cells are finally resuspended in phosphate-buffered saline (PBS) at a concentration of 3×10^6 cells/ml and maintained at 37°C until required. Cell viability as assessed by trypan blue dye exclusion was greater than 95% four hours after the final step in the cell preparation.

Aliquots of 0.75×10^6 lymphocytes per test (0.25 ml) are stimulated in disposable plastic tubes (Falcon No. 2003) by incubation for 45 min at 37°C after adding 50 μl of a 1/5 dilution of PHA (Gibco Europe). The stimulated cells are syringed to break up clumps and added to 3 ml of a test substrate containing 0.75 μg of fluorescein diacetate (FDA) (Riedel–De Haen, Hoechst U.K. Ltd). Microbial contamination of the substrate should be avoided, and the pH and osmolality are strictly controlled at 7.4 and 0.330 osmol/kg respectively. The test samples are transferred immediately to a 1-cm fluorescence grade quartz cuvette and placed in the thermostatted compartment (27°C) of a Perkin–Elmer MPF4 spectrophotometer equipped with polarisation facilities. The excitation beam (470 nm wavelength, 20 nm slit width) traverses a half-wave plate (Polaroid U.K. Ltd) to increase the fraction of the beam transmitted through the excitation polariser, which is set with its axis vertical. The fluorescein diacetate is converted to fluorescein inside the cells by enzymatic hydrolysis, and the fluorescent light at 90° is collected by the emission monochromator (510 nm wavelength, 20 nm slit width) and measured by a photomultiplier, using the ratio mode. The fluorescent light reaches the monochromator through a polariser which alternates between vertical and horizontal, thereby measuring the extent to which the vertical polarisation of the excitation beam has been retained in the fluorescent beam.

Each measurement is allowed to build up for a timed 6 min, after which the cells are quickly removed by gentle vacuum filtration (Millipore U.K. Ltd, 0.45 μm pore size) in order to measure and subtract the fluorescence levels contributed by leakage of the fluorescein back into the substrate. The polarisation index (P) of the sample is then calculated as described by the Cerceks [17].

To simplify the quantitative interpretation of results, the 'SCM Index' is defined [15] as the response from Zone 2 minus the response from Zone 1, where 'response' means the percentage reduction in the polarisation index after stimulation by PHA for 45 min at 37°C. A positive value for the SCM Index indicates that a greater response to PHA occurred in the lymphocytes from Zone 2, and is associated with malignant disease, whereas a negative SCM Index from non-cancer subjects indicates that the greater response to PHA occurred in the lymphocytes from Zone 1.

The simple SCM Index defined above fails to discriminate between two quite different clinical situations which both give rise to low values. A low Index can result from a low response in both zones, usually from patients with advanced cancer, where lymphocyte response to mitogenic stimulation is diminished by the large tumour burden. A low Index can also arise when both zones show large but nearly equal responses. This is to be expected from subjects whose SCM Index is changing polarity. With more experience, it should be possible to insert additional terms into the definition of the SCM Index to take account of the clinical significance of the two different ways in which low results can arise. For this report, we have chosen rather arbitrarily to consider all results in the range +4.9 to −4.9 as 'inconclusive'. Occasional increases in 'P' following PHA stimulation have been interpreted as 'non-responses' and are therefore not included in the calculation of the Index. Usually this has a negligible effect, but may eventually be shown to have some biological significance. Up to the present, cancer is the only disease which gives a positive result in the modified SCM test.

RESULTS

In this paper we present the results obtained from three groups of patients.

Lung series

Blood samples were taken from 29 male and 15 female patients with benign disease of the respiratory system and from 35 male and 5 female patients with lung cancer. The benign diseases were bronchitis (15, Group 1 in Fig. 1), asthma (4, Group 2), sarcoidosis (15, Group 3)

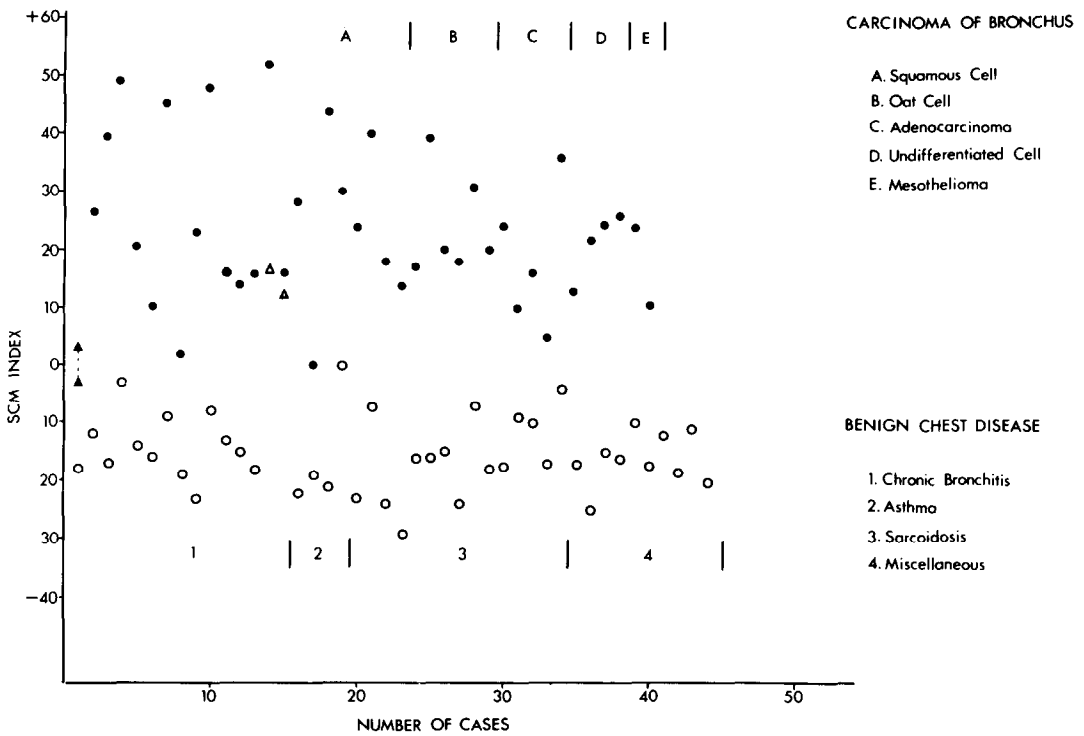

Fig. 1. SCM Indices from 84 patients with malignant (closed symbols) and non-malignant (open symbols) diseases of the respiratory system.

and a miscellaneous section (10, Group 4) which included lung abscess, bronchiectasis, Legionnaires disease, lung fibrosis, myocardial infarction, alveolar proteinosis, Hermansky Pudlak syndrome and active tuberculosis. The age range was 25–78 yr, with a mean of 49.3 yr. In the benign disease group 22 of the 44 (50%) had a history of smoking.

The cancer group consisted of squamous cell tumours (23, Group A), oat cell tumours (6, Group B), adenocarcinoma (5, Group C), undifferentiated tumours (4, Group D) and mesothelioma (2, Group E). The age range was 32–78 yr, with a mean of 61.5 yr, and 27 of the 40 (68%) had a history of smoking. Although this is not designated a true 'blind' series, in most instances the clinical diagnosis was unknown to the operator when the sample was measured. Except in one instance (see below), all SCM measurements reported in this paper were made before the start of any therapy.

The results from this series are shown in Fig. 1. A negative SCM Index of −3.5 was obtained from one patient (closed triangle) who had been treated by radiotherapy 5 years previously for a squamous cell carcinoma of the bronchus, but who was free from the disease, in the opinion of the clinician, at the time of the test. It has already been shown that the PHA response in Zone 1 returns after successful surgery or radiotherapy [3, 17]. A second test on the same patient 11 months later gave an SCM Index of +3.5 and revealed an abnormally high white cell count. Subsequent clinical examination showed chronic myeloid leukaemia. Two other cancer patients, as well as the one just mentioned, and two with benign diseases gave low results in the 'inconclusive' range from +4.9 to −4.9. In order to ascribe a level of confidence to the results, one patient with sarcoidosis was tested 5 times over a period of 9 months and gave SCM Indices in the range −22 to −28.

In the chronic bronchitis group (mean age 56.7 yr and 12/15 smokers) two positive results were obtained (open triangles). Nine months after the original tests one of these patients died from a myocardial infarction; a post-mortem examination was not carried out. The second positive patient has been re-examined by bronchoscopy and although some abnormalities of the bronchial mucosa were seen, malignancy has not been proved.

Leaving aside these 5 'inconclusive' results and the two positives for which histology is not available, all the remaining 77 results from this lung series were in agreement with the clinical findings.

Blind series

Results have already been published [15] from a small blind series of 20 coded samples

measured as part of the work to establish the significance of the differential PHA response of Zone 1 and Zone 2 lymphocytes. Here we report the results from a new larger blind series of 78 subjects, most of whom had diseases of the gastro-intestinal tract, respiratory system and breast. Blood samples were taken by the clinicians at three different hospitals separated by 3–10 miles from this Centre and code numbers were allocated to each sample. In most of the cases the clinical diagnosis had not been made at the time of the test, but was established some considerable time after the SCM result had been filed. On completion of the trial, which was spread over some 6 months, the SCM results were decoded and compared with the clinical diagnoses in the presence of the clinicians responsible for providing the original samples. At no time during this trial was there any contact between the test operator and the clinicians who supplied the coded samples.

The results from the 78 blind samples are shown as three main groups in Table 1 and summarised in Table 1(A). This trial was designed to check the discrimination between malignant and non-malignant diseases under clinical conditions, and therefore it was not planned to include controls as part of the blind series, but 4 healthy donors were added to the gastro-intestinal group by the clinician who provided the blood samples without the knowledge of anyone in the testing laboratory. Six cases were not tested because insufficient lymphocytes were recovered from the gradients. In normal clinical situations repeat samples would be requested from these patients, but in the blind trial it was considered unwise to complete a test and record a result when the cell yield was low.

Only two negative results were obtained from the patients with histologically proven malignant disease who were tested in this blind series. One of these (case No. 70) had advanced cancer where SCM is least reliable, leaving only one result (case No. 24) where SCM is clearly in disagreement with the histological findings. The positive rate was 12.5% in those patients who are at present considered on clinical grounds to be free from malignant disease. In 10 cases low but not necessarily incorrect results were obtained in the range at present considered inconclusive. All of the 6 cancer patients in this last group had advanced malignant disease which usually causes low PHA responses from both zones and therefore a low SCM Index.

Cumulative clinical results

In addition to the 162 subjects in the two series just described, 202 other patients with confirmed malignant disease were tested over the past two years as part of the continuing assessment and development of the modified SCM test. These were not measured blind, and the results are summarised in Table 2. The

Table 1. *A blind study of the modified SCM test in malignant and non-malignant diseases at three sites*

Sample	Sex	Age	'P' values—Zone 1 U	'P' values—Zone 1 PHA	'P' values—Zone 2 U	'P' values—Zone 2 PHA	I_{SCM}	Diagnosis
					Gastro-intestinal tract			
1	M	73	0.204	0.183	0.230	0.192	+6	Cancer, stomach
2	M	48	0.168	0.165	0.209	0.189	+8	Benign, cholecystitis
3	M	60	0.181	0.171	0.181	0.115	+31	Cancer, colon
4	F	82	0.174	0.165	0.225	0.225	−5	Benign, oesophageal stricture
5	F	64	0.168	0.158	0.202	0.144	+23	Oesophageal cancer
6	F	28	0.169	0.125	0.164	0.150	−17.5	Healthy donor
7	M	71	0.166	0.163	0.186	0.143	+21	Cancer, sigmoid colon
8	M	52	0.177	0.186	0.231	0.144	+38	Cancer, transverse colon
9	M	60	0.187	0.180	0.181	0.146	+15.5	Cancer, rectum
10	M	73	0.164	0.163	0.182	0.143	+21	Cancer, head of pancreas
11	M	74	0.187	0.172	0.191	0.176	0	Cancer, stomach, advanced
12	F	18	0.172	0.144	0.179	0.171	−12	Healthy donor
13	M	40	0.179	0.172	0.203	0.180	+7	Cancer, rectum
14	M	72	0.159	0.146	0.171	0.145	+7	Cancer, stomach, advanced
15	F	20	0.156	0.118	0.177	0.157	−13	Healthy donor
16	F	70	0.179	0.160	0.173	0.127	+16	Cancer, colon
17	F	41	0.172	0.138	0.224	0.128	+23	Cancer, rectum
18	M	62	0.183	0.171	0.212	0.149	+23	Benign, diabetic abscess
19	F	26	0.158	0.128	0.183	0.182	−18.5	Healthy donor
20	M	56	0.163	0.154	0.167	0.121	+21	Benign, rectal polyp
21	M	72	0.189	0.171	0.197	0.178	0	Cancer, stomach, advanced

Table 1. (*Contd.*)

Sample	Sex	Age	'P' values—Zone 1 U	'P' values—Zone 1 PHA	'P' values—Zone 2 U	'P' values—Zone 2 PHA	I_{SCM}	Diagnosis
					Gastro-intestinal tract			
					Lung			
22	M	62	0.183	0.155	0.184	0.114	+23	Squamous cell carcinoma
23	M	64	0.173	0.170	0.178	0.199	0	Squamous cell carcinoma, advanced
24	F	57	0.167	0.124	0.183	0.183	−25.5	Oat cell carcinoma
25	M	72	0.169	0.128	0.171	0.158	−16.5	Bronchiectasis
26	M	76	0.171	0.133	0.173	0.165	−17.5	bronchiectasis
27	M	62	0.160	0.155	0.185	0.147	+17.5	Squamous cell carcinoma
28	M	78	0.202	0.164	0.202	0.169	−3	Chronic bronchitis, cor pulmonale
29	M	61	0.182	0.149	0.188	0.177	−12	Pneumoconiosis, cor pulmonale
30	M	62	0.168	0.158	0.207	0.166	+14	Pernicious anaemia, cancer prostate
31	M	60	0.178	0.179	0.208	0.168	+19	Chronic bronchitis
32	M	55	0.188	0.165	0.203	0.157	+10.5	Asbestosis
33	M	37	0.175	0.142	0.196	0.103	+28.5	Squamous cell carcinoma, cervical glands
34	M	49	0.175	0.143	0.176	0.127	+10	Carcinoma lung, cervical glands
35	M	67	0.152	0.128	0.173	0.117	+21	Chronic bronchitis
36	M	73	0.152	0.160	0.157	0.145	+7.5	Squamous cell carcinoma
37	M	59	0.181	0.133	0.184	0.156	−11	Cryptogenic fibrosing alveolitis
38	F	61	0.179	0.157	0.160	0.177	−12	Cryptogenic fibrosing alveolitis
39	F	54	0.167	0.174	0.183	0.150	+18	Squamous cell carcinoma
40	M	59	0.178	0.151	0.209	0.201	−11	Chronic bronchitis
41	F	64	0.173	0.128	0.188	0.173	−18	Chronic bronchitis
42	M	66	0.182	0.169	0.207	0.186	+3	Squamous cell carcinoma, advanced
43	M	71	0.181	0.162	0.187	0.152	+8	Poorly differentiated cell carcinoma
					Breast			
44	F	29	0.178	0.125	0.164	0.150	−21.5	Benign, fibroadenosis
45	F	50	0.170	0.173	0.199	0.164	+17.5	Cancer, recurrent
46	F	48	0.199	0.191	0.198	0.160	+15	Cancer
47	F	36	0.190	0.127	0.163	0.160	−31	Benign, fibroadenosis
48	F	32	0.179	0.152	0.171	0.112	+20	Benign, ductectasia
49	F	43	0.222	0.127	0.217	0.160	−16.5	Benign, fibroadenosis
50	F	44	0.164	0.131	0.160	0.151	−14.5	Benign, fibroadenosis
51	F	57	0.190	0.158	0.179	0.149	0	Cancer, Stage IV
52	F	43	0.157	0.147	0.166	0.138	+10.5	Benign, fibroadenosis
53	F	49	0.215	0.183	0.233	0.179	+8	Cancer Stage IV
54	F	70	0.192	0.167	0.246	0.171	+17.5	Cancer, Stage III
55	F	32	0.191	0.137	0.188	0.151	−8.5	Benign, fibroadenosis
56	F	32	0.165	0.135	0.173	0.148	−4	Benign, fibroadenosis
57	F	41	0.169	0.141	0.166	0.167	−16.5	Benign, fibroadenosis
58	F	26	0.165	0.117	0.177	0.155	−17	Benign, fibroadenoma
59	F	49	0.168	0.141	0.166	0.146	−4	Benign, fibroadenosis
60	F	36	0.197	0.134	0.188	0.160	−17	Benign, fibroadenosis
61	F	44	0.178	0.134	0.190	0.174	−16	Benign, fibroadenosis
62	F	42	0.199	0.201	0.220	0.230	0	Cancer, Stage IV
63	F	26	0.185	0.138	0.152	0.185	−25	Benign, fibroadenosis
64	F	20	0.160	0.126	0.173	0.170	−19	Benign, fibroadenosis
65	F	34	0.182	0.163	0.182	0.172	−5	Benign, ductectasia
66	F	47	0.183	0.174	0.174	0.152	+8	Cancer, Stage III
67	F	27	0.166	0.164	0.175	0.157	+9	Benign, fibroadenoma
68	F	40	0.161	0.180	0.168	0.169	0	Benign, fibroadenosis
69	F	30	0.151	0.115	0.161	0.162	−24	Benign, fibroadenosis
70	F	63	0.184	0.160	0.209	0.200	−9	Cancer, Stage IV
71	F	41	0.178	0.135	0.204	0.197	−21	Benign, fibroadenoma
72	F	53	0.197	0.189	0.198	0.153	+19	Cancer, Stage III

Six samples were not measured because the cell numbers were too low.
U = unstimulated 'P' value. PHA = 'P' value after stimulation with PHA.

Table 1(A). Summary of the results from the blind study

	GI Tract	Lung	Breast	Controls	Total
Number of cases tested	17	22	29	4	72
False negatives	0	1	1	—	2(2.8%)
False positives	3	3	3	0	9(12.5%)
Inconclusive (within range +4.9 to −4.9)	2	3	5	0	10

Six samples were not measured because the cell numbers were too low.

figures quoted for the range of the SCM Index in each disease group do not include the 5 negative results, which are tabulated individually to avoid confusion in the significance of the ranges. One of these negative results was from a patient who had been treated for Stage I cancer of the cervix, but at the time the blood sample was taken this patient was clinically free from disease and would be expected to give a negative SCM result. The other 4 negative results were in disagreement with the clinical findings.

Figure 2 shows the results from a preliminary attempt to establish the minimum tumour size needed for a positive SCM Index in a small series of 18 patients with basal cell carcinoma of the skin. Tumours 0.4 cm in diameter or more gave good positive results in our modified SCM test. That such a small skin tumour should give rise to a clear reversal of the Zone 1/Zone 2 response to PHA is difficult to explain, but not inconsistent with experiments in mice [18], where it was shown that as few as 6.5×10^5 implanted Ehrlich ascites tumour cells produced a marked SCM response.

DISCUSSION

The SCM test was first described 7 years ago [1], but its development as a clinically useful technique has been slow. Many of the early difficulties in reproducing the Cercek results were associated with the use of CaBP, which is now known to lose its activity in storage and to be specific for certain organ sites in this assay. It should be emphasised again that the SCM technique described by the Cerceks was based on the differential response of Zone 1 cells to PHA and CaBP, and therefore depended on the unstable characteristics of relatively crude tumour extracts. The modified double-zone SCM technique developed in these laboratories depends only on PHA, which maintains a stable activity within a particular batch. A fresh batch should be checked using a healthy donor who maintains a steady SCM Index in repeated tests, and should be discarded if it shows low activity. Increasing the concentration to restore activity results in cell aggregation, together with unacceptably high levels of fluorescence in the filtrates caused by increased permeability of the cell membranes.

It was thought at first that the double-zone cell separation was selecting a sub-population of lymphocytes which had suffered a density change and therefore settled in a different zone of the gradient [15]. There is some evidence, both in animal and human studies [19–22], to suggest that density differences can be found between sub-populations of lymphocytes However, careful experiments have shown that there is no measurable change in cell numbers between the two zones in cancer and non-cancer subjects [16] and we now assume that the test depends on functional changes in the sub-populations of lymphocytes.

A sensitive cancer test is not required to detect advanced malignant disease, and yet there is a tendency to use patients with advanced cancer in the early stages of the laboratory testing of any new technique such as SCM, or when subjects are selected for a blind trial by a 'neutral' clinician not associated with the testing laboratory. For example, all of the low results from cancer patients in the blind study summarised in Tables 1 and 1(A) are from patients with advanced cancer who would derive least benefit from a sensitive cancer test in clinical practice. It is an obvious advantage of SCM that its sensitivity and reproducibility are highest in detecting early cancer, as is demonstrated under somewhat limited conditions in the experiment leading to Fig. 2.

Some low results in populations of control or benign disease subjects are to be expected on the basis of cancer statistics, from those individuals who are in transition from negative to positive because of sub-clinical malignant disease. It is therefore not surprising that the

Table 2. Cumulative results from 202 cases of proven malignant disease

Site	Number/Sex	Age range (yr)	SCM Index Negative	Weak positive	Full positive	Range of full positive	Mean of full positive
Cervix	52F	27–75	1*(−16)	0	51	+6 to +34	+17.1
GI tract	15F 17M	39–83	2(−20) (−9)	2	28	+5 to +36	+17.4
Breast	19F–	34–78	0	1	18	+7 to +35	+19.1
Bladder	1F 8M	56–78	1(−8)	0	8	+9 to +37	+19.4
Skin > 0.4 cm	10F 14M	34–89	0	0	24	+7 to +50	+20.0
Bronchus	5F 61M	38–76	1(−15)	2	63	+6 to +49	+22.7

*Previously treated for carcinoma of cervix, no clinical evidence of tumour.

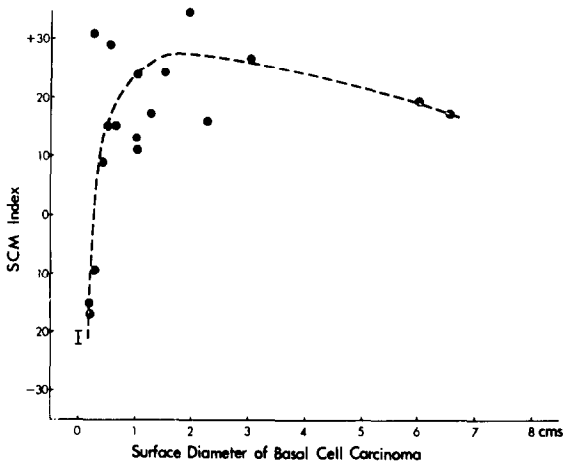

Fig. 2. Dependence of SCM Index on the surface diameter of the tumour for patients with basal cell carcinoma.

number of inconclusive results is high (6 out of 39) amongst the non-malignant subjects of Table 1, some of whom must represent a high-risk group for sub-clinical cancer because they are in the right age range and have conditions such as breast disease.

When the results from the three series are combined, omitting the 22 inconclusive results for the reasons already stated, this study shows an overall rate of 1.8% 'false' negatives and 3.3% 'false' positives in a total of 336 blood samples measured in this laboratory by the modified double-zone SCM technique. Our results demonstrate that the technique can be used with confidence as an aid to the clinical diagnosis and monitoring of malignant disease.

Acknowledgements—We thank Tenovus and South Glamorgan Health Authority (Teaching) for generous financial support.

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