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Original Paper

Adaptation of the Cellscan Technique for the SCM Test in Breast Cancer

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The value of the SCM (Structuredness of Cytoplasmic Matrix) cancer test, a procedure based on the detection of differences in lymphocyte activation in the presence and absence of cancer, has remained controversial, with inconsistent results having been reported among investigators. The Cellscan, a high-precision static cytometer system, has been designed to perform the SCM test; the apparatus facilitates the polarisation measurements and can examine cells which have been separated by simpler procedures than were originally described. In this study, using methods and diagnostic criteria adapted for the Cellscan system in a hospital environment, the SCM test correctly classified over 90% (76/80) of patients with breast cancer and differentiated over 90% (72/73) of individuals without cancer.

Key words: cancer detection, immune system, fluorescein fluorescence polarisation, lymphocytes, SCM test

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INTRODUCTION

As a potentially simple and rapid test to discover malignancy at an early stage, the application of intracellular fluorescein fluorescence polarisation (IFFP) measurements to detect lymphocyte sensitisation first reported by Cercek and Cercek [1] caused widespread interest. They demonstrated that lymphocytes from cancer patients showed a 20% decrease in their polarisation value after exposure to tumour-derived antigen preparations such as cancer basic protein (CaBP) or encephalitogenic factor (EF), but not to phytohaemagglutinin (PHA), while lymphocytes from healthy donors showed a similar response to PHA but not to EF or CaBP [2]. Thus, a general yes–no result is given by the ratio of the degrees of polarisation after the two manipulations. This is expressed by the value of $RR_{SCM} = P_{EF}/P_{PHA}$. An $RR_{SCM} < 1$ definitively indicates a state of malignancy while an $RR_{SCM} > 1$ rules it out. Moreover, incubation of the lymphocytes with a tumour tissue or an extract of a tumour (or of a tumour cell culture, or of the supernatant of such culture) will reduce the degree of polarisation of the donor of the lymphocytes if he suffers from a cancer of the same organ origin as that allogeneic extract. The affected organ of the patient can thereby be determined [1, 3–5]. This procedure, originally devised and modified by Cercek and Cercek [1] and others [6–8], is the Structuredness of Cytoplasmic Matrix (SCM) test. Since some researchers [3, 6, 9–16] succeeded in fully or partially repeating the test results while others [17–21] experienced varying degrees of technical difficulties, the SCM test became rather controversial.

We succeeded in confirming the validity of the SCM method in our laboratory in approximately one thousand cases [4, 5, 7, 22]. Our experience shows that success in using the original SCM test can be easily thwarted and that almost any deviation in adhering to the strict protocol prescribed by the Cerceks can result in failure.

We recently described an alternative apparatus and methodology called Cellscan, which permits the repetitive measurement of IFFP in individual cells subjected to a variety of manipulations [8]. We now discuss the use of this technology to perform a cytometric equivalent of the SCM test.

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The heart of the Cellscan system is the cell carrier (Figure 1). It provides a means of maintaining an ensemble of cells in fixed positions in individual traps (Figure 2) while permitting changes to be made in their micro-environment. The present study was designed to assess the capability of the Cellscan system to diagnose breast cancer. The sensitivity and specificity of prospective Cellscan testing of patients with undiagnosed breast masses, performed in two breast clinics at university medical centres, are described in detail.

MATERIALS AND METHODS

Apparatus

The central feature of the Cellscan system [8] is the cell carrier incorporating a 100 by 100 two-dimensional array of holes, each approximately 7 μm in diameter and spaced approximately 20 μm apart, in which cells are trapped. The carrier is fixed to a holder which allows the introduction and extraction of liquids during which cells are maintained in their individual traps, permitting observation of the cells while changes occur in the cellular micro-environment.

A 40 μl aliquot of cell suspension is applied to a carrier; a negative pressure of approximately 1–5 mm H_2O maintained between the upper and lower surfaces of the carrier causes rapid settling of the cells into traps as described elsewhere [8].

Measurements of fluorescence with the Cellscan employ a photon-counting technique in which the same number of photons is counted for each cell, regardless of intensity, so that strongly emitting and weakly emitting cells are measured with the same precision. Measurement of background signals due to dark current, stray light and/or autofluorescence can be made for each cell prior to its fluorescence measurement.

The cell carrier holder is fastened to a scanning table driven by three stepping motors; a motion in the x–y plane will position any trap in the cell carrier in the centre of the epi-illuminating laser beam and the microscope observation field, while focus is changed by the z-axis motor. Under the prescribed staining conditions and with illumination intensities of 1–5 μW at 441 nm, the sampling time for obtaining counts of 10000 photons from a single fluorescein-loaded cell varies from 0.05 to 1 second. The coordinates of each cell, its fluorescent intensity (expressed in number of counts per unit time) at two different wavelengths (527 nm and 510 nm), the counting time, and the computed polarisation values were recorded, as were the mean, standard deviation, coefficient of variation (CV) and number of good and bad (off limit) values for each parameter measured during the run.

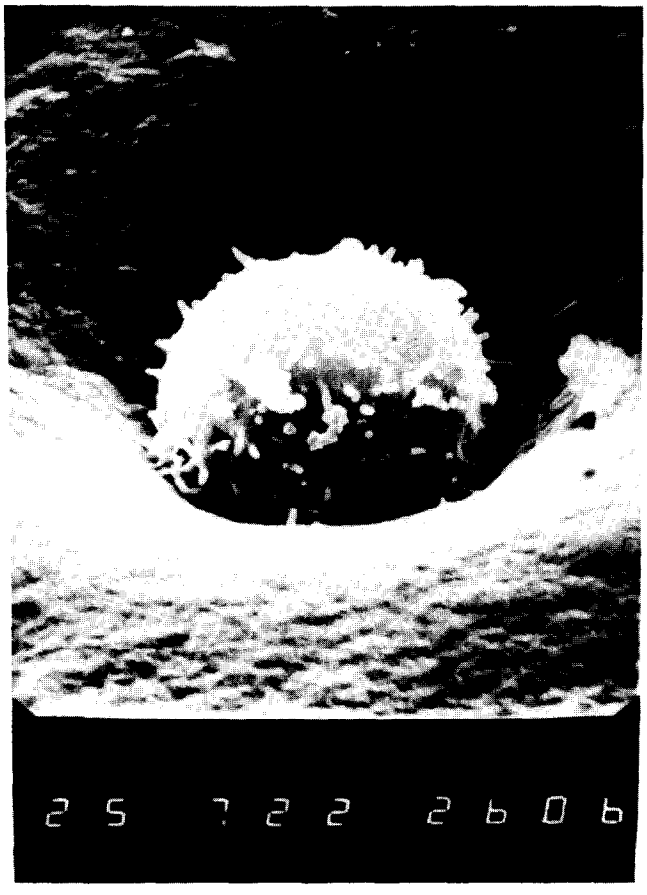

Figure 2. A lymphocyte after settling down in a trap.

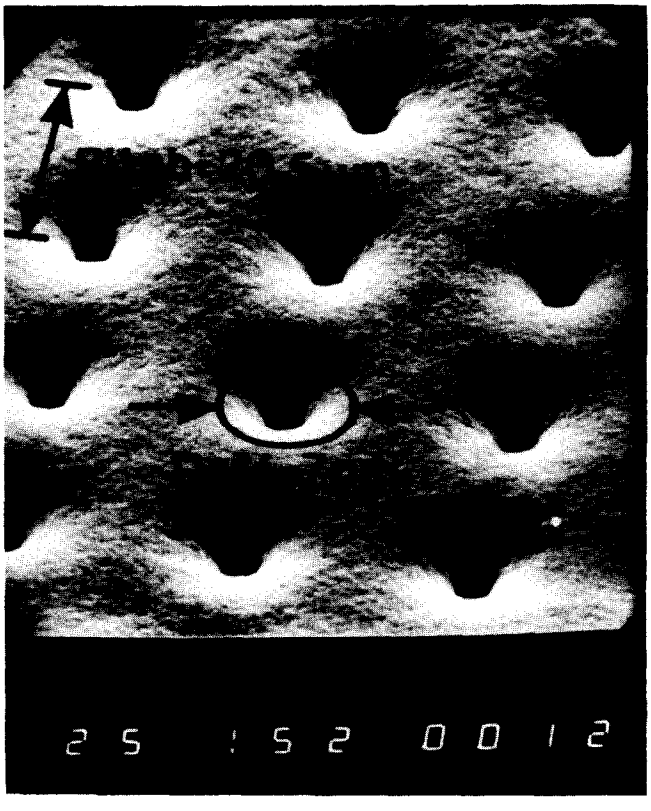

Figure 1. A section of the cell carrier.

Blood samples

Participating in this study were 216 subjects, of whom 143 women (median age 59 years, range 31–82 years) with palpable breast masses were admitted to the breast cancer clinics at the Tel-Aviv and Beilinson University Medical Centres in 1993. All subjects underwent physical examination, mammography and biopsy: 80 had breast cancer and 63 were cancer free.

The control group was comprised of 73 volunteers who were asymptomatic individuals and patients hospitalised for non-malignant disease.

Excluded were women who still had effects related to recent chemotherapy, anaesthesia, radiotherapy and those women who were 3 days before, after or during menstruation, or pregnant.

Solvents and markers

The preparations of modified Dulbecco PBS (330 mosm/kg and 1.2 mM Ca^{2+}), modified Ficoll-metrazoate gradient solution for cell separation and FDA colouring solution have been described previously [7].

Reagents

Two groups of stimulants were used in the SCM assay: (a) Non-specific reagents: PHA (Phytohaemagglutinin, Wellcome, U.K.) and EF (Encephalitogenic Factor, Peninsula, U.S.A.), which are commercially available. (b) Specific reagents: tumour antigen extracts (TAE) which were used for organ malignancy determination. The preparation of the calibrated TAE was as follows: pieces (1–3 g) of tumour tissue (Infiltrating Duct Carcinoma) or pellets of tumour cell lines (MCF-7, T47D) were thoroughly rinsed several times in PBS, suspended in 5 volumes (v/v) of 3 M KCl solution (pH = 7.4) and homogenised. The homogenate was stirred overnight at 4°C and then centrifuged at 20000*g* for 30 min at 4°C. The supernatant was collected and the KCl was removed by gel filtration through a Sephadex G-25 column; the eluted solution was precipitated with 2 M (final concentration) ammonium sulphate for 1 h at 4°C and centrifuged for 30 min at 20000*g* and 4°C. The precipitate was collected and suspended in double distilled water. To remove the ammonium sulphate, the suspension was gel-filtrated through a Sephadex G-25 column and the eluted solution was lyophilised. For practical reasons, the lyophilised powder was diluted in PBS at a concentration of 10 mg/ml and stored at −90°C. The optimal concentrations of each of the stimulating extract solutions were found empirically. The most commonly and successfully used TAE was the T47D preparation.

Lymphocyte separation

Twenty millilitres of peripheral blood was drawn into heparinised (600 units) syringes from each participant. Lymphocytes were separated as follows: 0.1 g carbonyl iron powder was added to 10 ml heparinised (300 units) peripheral blood, rotated at 15 RPM at 37°C for 30 min and then placed on a magnet for 15 min at 37°C for sedimentation of the iron-absorbing cells. This was followed by gentle suction of the full blood, taking care not to collect the sediment. From this stage on, two modes of separation were used in hospital laboratories:

Mode (a). One millilitre of four parts lymph-Prep (LP) (Nycomed, Sweden) or Ficoll-Paque (FP) (Pharmacia, Sweden) gradient solutions, diluted with one part of saline, were carefully laid on 3 ml LP or FP in a 10-ml glass tube to which 4 ml of phagocyte-depleted blood was gently added. After centrifugation, the layer obtained at the interface between the diluted and undiluted gradient was collected by a Pasteur pipette. After collection, cells were washed twice in saline and once in PBS, and then suspended in PBS at 10^7 cells per ml. This mode was used in most of the cell separations in this study.

Mode (b). This is the original Cercek procedure in which a modified Ficoll–Triozil (Metrazoate) gradient was applied [1].

Loading of cells with fluorescein

This was done by fluorochromasia [23] which could be carried out either before or after loading of the cells on the cell carrier. Before loading, 20 μl of cells were mixed with 20 μl of 1.2 μM FDA (fluorescein diacetate) (Riedel De Haen, Germany) to reach a final FDA concentration of 0.6 μM. After 4-min incubations with FDA, the cells were placed on to the cell carrier and the FDA solution was gently washed away with PBS. After loading, FDA 0.6 μM was directly applied on to the cell carrier, and after 4 min, the cells were rinsed by PBS and further colouring was discontinued.

Stimulation

Stimulation was achieved by adding one aliquot of stimulant to nine aliquots of cell suspension. In the majority of the measurements, stimulant application was performed in the first of the two alternative modes: I. Stimulation of cells was carried out with the cells in suspension. In this case, both control and stimulation measurements were carried out on different cell carriers. II. After the control measurements, a drop of the stimulant solution was added to the cell-containing carrier and incubation at 37°C was induced while the cells were on the apparatus. During the incubation, the Cellscan was, of course, idle. In some cases, the carrier holder was removed from the apparatus, placed into an incubation chamber for the period of stimulation, and then replaced on the apparatus. In both of the above modes, gentle agitation of the cells can be applied for better contact with the stimulant solutions. The incubation times were 45 min for PHA and 30 min each for EF and TAE.

RESULTS

Calibration of polarisation measurements and measurement precision

Calibration was carried out as follows [8]. First, the polarisation measured by the Cellscan was compared with calculated polarisation values using a white light source. Second, the polarisation values of various viscosity solutions of fluorescein in glycerin water mixtures obtained by the Cellscan were compared with those measured by a Perkin-Elmer MPF-44 and SLM-4800 polarisation fluorimeters. Third, the polarisation of fluorescent beads as measured by a fluorimeter was compared with that measured by the Cellscan. Fourth, the previous procedure was repeated with living cells. All polarisation measurements were carried out at a controlled room temperature of 22 ± 1°C. Fading of the fluorescence is insignificant under the measurement conditions and, in any event, does not influence the polarisation (Deutsch *et al.*, manuscript in preparation).

Persistence and reproducibility measurements

Persistence and reproducibility were tested by 25 routine cell scans of a field of 10 × 10 traps for beads and by 10 scans for cells. For each bead or cell, the persistence on the carrier and spread of polarisation as well as the coefficient of variation (CV) were determined. The results are presented in Figure 3, parts I, II and III, respectively. Although the particles were rinsed throughout the scans, no bead was lost (the lengths of all the persistence lines are equal) and the majority of the cells also survived the ten measurements. The spread of polarisation values is shown by the length of the line (Figure 3). The CVs for individual beads never exceeded 3% and some were much lower. The distribution of polarisation values for cells was appreciably wider and the differences between cells were also considerably greater than those between beads. Nevertheless, the average CV for the individual cells did not exceed 2.75%.

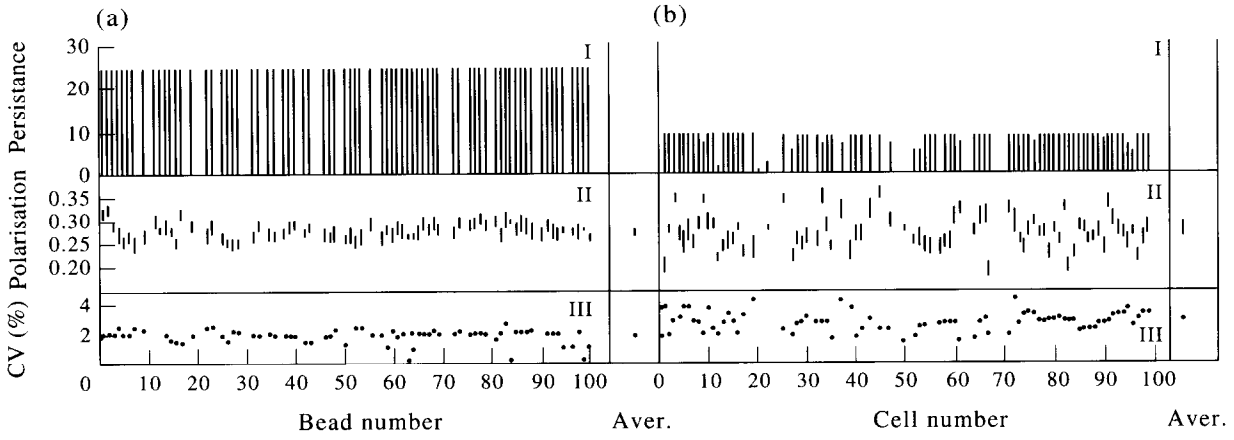

Figure 3. (I) Persistence of particles; (II) Spread of polarisation values; (III) Spread of polarisation CV. (a) For 25 scans of beads, (b) for 10 consecutive scans of cells. Larger spaces between the lines indicate an unsettled trap.

The mean IFFP value of lymphocytes before stimulation, representing an average of mean values of cells on over 600 carriers from over 200 individuals with and without cancer, was 0.203 ± 0.012 (CV approximately 6%); mean values computed separately for patients and controls were not notably different ($P > 0.90$). The CV for prestimulation measurements of cells on an individual carrier is typically approximately 10%.

Data collection and display: representative examples

For this study, a total of 216 samples were analysed: 73 from a control group (asymptomatic individuals and patients without malignant disease) and 143 from patients with a palpable and undiagnosed tumour of the breast. Measurements were carried out in unstimulated control samples as well as on cells stimulated with PHA, EF and, in most cases, one or more tumour antigen extracts (TAEs). Representative data are shown in Figure 4.

Each panel in Figure 4 is a scattergram in which the x and y coordinates of individual cells respectively represent polarisation values measured before (P_b values) and after (P_a values) stimulation. Figure 4a shows P_b and P_a polarisation values of unstimulated cells from a patient with breast cancer and Figure 4b shows P_b/P_a values of cells from the same patient following stimulation with a breast cancer antigen extract (Br-203). No stimulant had been added between the measurements presented in Figures 4b and c. The difference between the polarisation values merely demonstrates that the stimulation process continued and expressed itself in the more reduced polarisation values.

The average P_b and P_a values in Figure 4 (designated by B and A), and cumulative distributions of the ratio R from 1.0 to 1.3 in steps of 0.1 of P_b and P_a values, are tabulated in the upper left hand corner of each panel. The broken lines on each side (at 45°) of the solid line define the region in which P_a values differ from P_b values by less than 10%.

In the example depicted by Figure 4b, a significant decrease in polarisation following stimulation with tumour antigen, indicated by changes in the means and differences in the cumulative distributions, is observable on the scattergrams. However, differences less obvious to the eye may still be statistically significant. A comparison between the tabulated data in Figure 4a and b indicate stimulation. The percentage of cells having an $R > 1$ changed from 50 to 73%, while those having an $R > 1.1$ increased from 12 to 36% and those with an $R > 1.2$ from 2 to 13%. These figures convey information not only on the depth of the stimulating effect of the cancer reagent, but also on its kinetics (Figure 4b, c), as well as on the distribution of the lymphocytes with regard to these effects. The relatively small contribution of instrumental factors to variance is demonstrated by measurements performed on fluorescent beads (Figure 5a). The P_b and P_a values are minimally dispersed about the 45° line, as compared with the cells.

Figures 5b and c are representative examples of results obtained after cell stimulation in suspension. Figure 5b shows the histogram of the polarisation values for the lymphocytes of a healthy individual which have been incubated with EF (encephalitogenic factor). EF did not stimulate the lymphocytes and the histogram did not change.

Figure 5c shows the histogram of the polarisation values for the lymphocytes of the same healthy individual which had been incubated with PHA. The lymphocytes became stimulated, as is seen by the shift of the P_{PHA} histogram, to the lower polarisation values with regard to the full line contour of the P_c histogram of the control values.

A representative example which demonstrates the relative constancy of polarisation values for cells from a single individual obtained by the Cellscan is illustrated by Figures 6a and b. These show distributions of the quantity $(\overline{B} - B)/\overline{B}$, where B is the mean polarisation of cells on a single carrier from a single individual, and $\overline{B}$ is the mean of B values from several carriers from the same individual. The data from controls (in Figure 6a) and those from patients with cancer (Figure 6b) are comparable.

There is, however, a notable difference between controls and cancer patients in response to stimulation with EF or TAE, as shown in Figures 6c–f. The distributions of values of $(B - A)/B$, i.e. the relative change in polarisation following stimulation, in controls (Figure 6c) and patients (Figure 6d) reflect the generally greater changes observed in the patients (negative changes are not considered).

Figures 6e and f are based on measurements replicated 2–5 times. Figure 6e, like Figure 6c, shows relative changes in polarisation in cells from controls following stimulation with EF or TAE. The quantity plotted in Figure 6e, however, is $(\overline{B} - A_{min})/\overline{B}$, where $\overline{B}$, as before, is the mean of mean prestimulation values from several carriers, while A_{min} is the

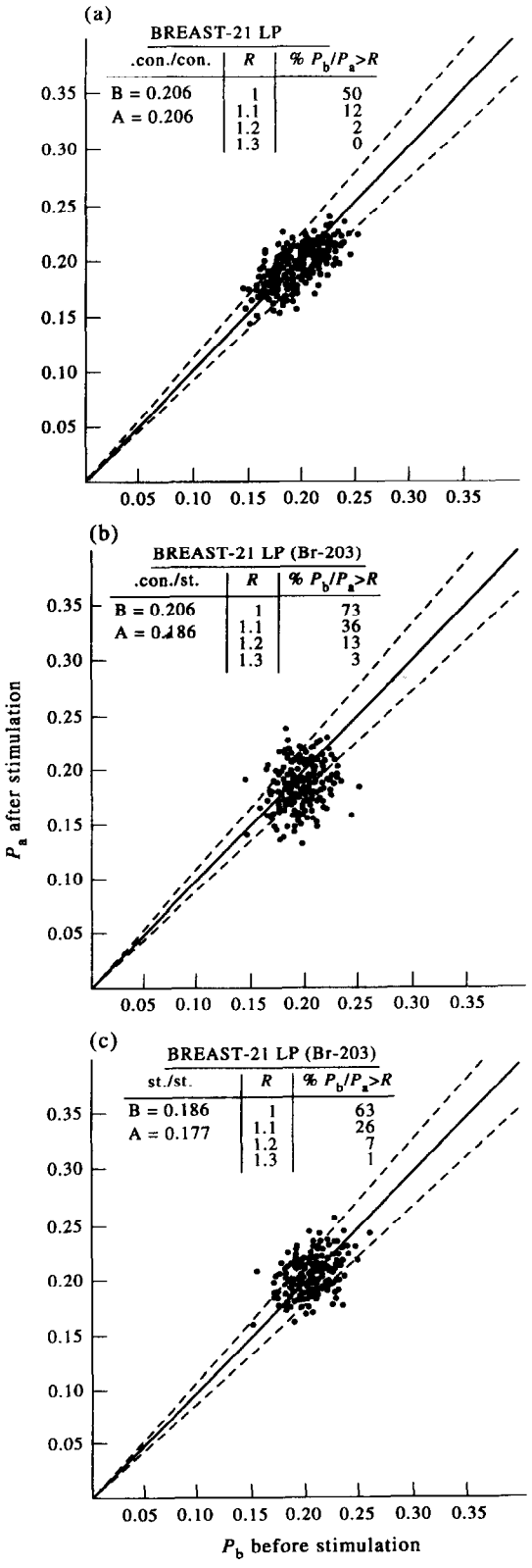

Figure 4. A representation of the Cellscan results. Each point in the figure represents the polarisation of one cell: (a) two consecutive control measurements; (b) stimulation with breast TAE; and (c) continuing stimulation after incubation with stimulant. The average P_b and P_a values (inserts B and A) and cumulative distributions of the ratio R from 1.0 to 1.3 in steps of 0.1 of P_b/P_a values are tabulated in the upper left hand corner of each panel. The broken lines on each side (at 45°) of the solid line define the region in which P_a values differ from P_b values by less than 10%. Cells were separated by LP.

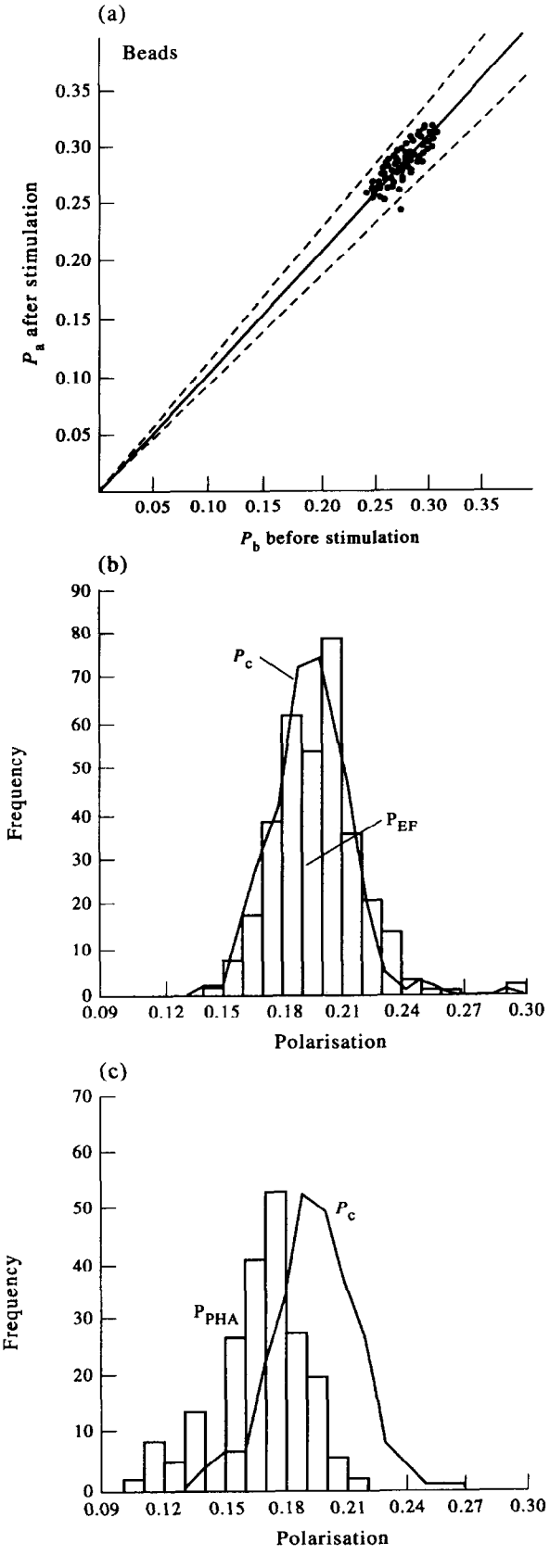

Figure 5. (a) Two consecutive fluorescence polarisation measurements of beads; (b) histogram of P_c (control values) and of P_{EF} after incubation with EF (healthy individual); and (c) histogram of P_c (control values) and of P_{PHA} after incubation with PHA (same healthy individual). Stimulation of cells was carried out in suspension.

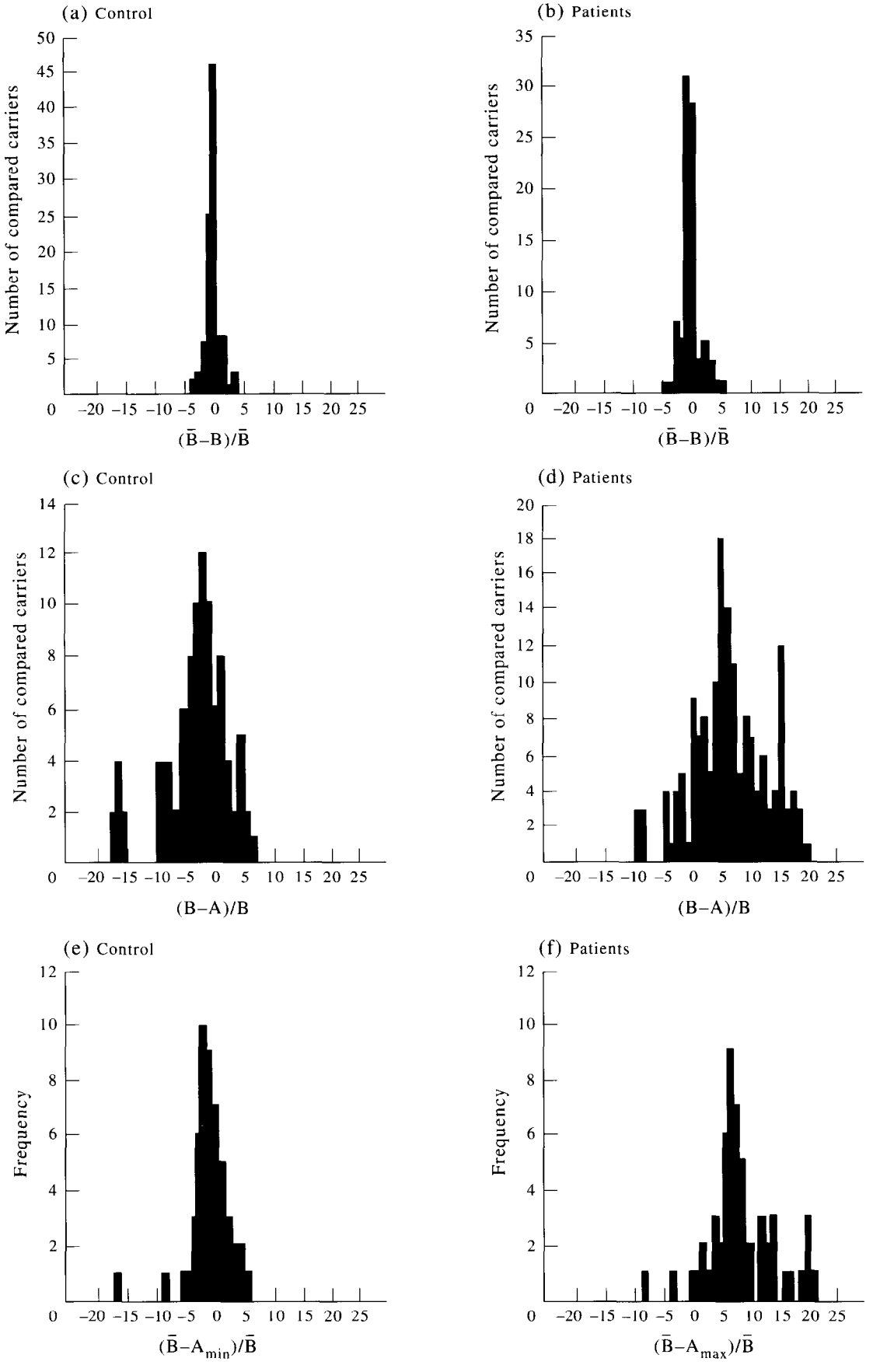

Figure 6. Distributions of computed parameters, described in the text, for controls: (a), (c) and (e); and cancer patients: (b), (d) and (f).

lowest mean polarisation value observed among the replicates, i.e. the value representing the greatest change in polarisation following stimulation. Data from patients are plotted in Figure 6f; in this case, the poststimulation value used is A_{max}, i.e. the value representing the smallest change in polarisation following stimulation. Even when the histograms are thus intentionally biased in a way which should minimise the difference between controls and cancer patients, the more pronounced changes in patients are clearly discernible.

Effects of lymphocyte separation procedure

The Ficoll–Metrazoate lymphocyte separation method originally used by Cerceks and associates [1] is cumbersome and time consuming; accordingly, we examined simpler preparative procedures commercially prepared Lympho-Prep (LP) and Ficoll-Paque (FP) solutions. Cells separated using the Cerceks' procedure consistently showed larger changes in polarisation following stimulation; in analyses of replicate samples from the same donors, we found that the average decrease in polarisation was 10 ± 4% in cells separated by LP or FP and 18 ± 8% in cells separated by the Cercek method.

Development of provisional diagnostic criteria

The following criteria were used to arrive at provisional diagnoses for the 216 samples collected from patients and controls. As represented by the ratio of the average polarisation value obtained after stimulation with EF and that obtained after stimulation with PHA, a sample was considered 'cancer-positive' when RR_{SCM} (P_{EF}/P_{PHA}) was <1, or when RR_{SCM} was >1 and the polarisation was decreased by at least 5% in response to any TAE.

The reason for $RR_{SCM} > 1$ in cancer patients is the faulty/deficient/defective collection of the 'lower band' cells, located between 2 mm and 1.5 cm below the interface from the gradient separation tube. This might occur erroneously or if these cells were initially mixed with the cells located in the interface. In such cases, a response to PHA is expected [24]. In order to prevent a 'cancer-positive' decision in such cancer patients, a decrease of 5% in the response to TAE is used also as an indication for 'cancer-positive'.

A sample was considered 'cancer-negative' if RR_{SCM} was >1 and/or no TAE decreased polarisation by at least 5%. The results, broken down by tumour types and stages, are shown in Table 1.

When the above criteria were applied, the Cellscan correctly classified 95% of samples from patients with breast cancer as being positive, and 98.6% of samples from individuals without breast cancer as being negative. Of the 95% of the patients found to be pathologically positive for breast cancer, the Cellscan results were not correlated to the stage of disease. All 63 women who ostensibly had benign tumours had positive findings on mammography, while their pathological findings were negative. Within the same period of time, blood samples were taken from these women and examined by the Cellscan apparatus: 79.4% (50/63) had negative Cellscan results, while those of the remaining 13/63 were positive. During an 18-month follow-up period, 30% (4/13) of the latter were eventually discovered to have carcinoma of the breast, as confirmed by pathological findings.

DISCUSSION

The clinical results reported here document the first application of high-precision cytometry to the performance of the SCM test which was carried out in both hospitals in parallel with the conventional workup. While these results are promising, a considerable amount of work will be required to establish the diagnostic utility of both the SCM test and the Cellscan system. The apparatus simplifies and increases the precision of the fluorescence polarisation measurements required for the SCM test and allows the analysis to be made on lymphocytes isolated by simpler procedures than those originally prescribed by Cerceks and associates.

Both flow and static cytometry can be used to obtain estimates of the fraction of activated lymphocytes in a cell population. However, only static systems such as the Cellscan can presently define the time course and magnitude of response to stimulation in individual cells. This capacity, in our opinion, makes the Cellscan the apparatus of choice for performance of the SCM test; the kinetic information we can obtain should also be useful in understanding the process of lymphocyte activation and have clinical relevance as well.

In the present study of 143 women with suspected breast cancer, peripheral blood samples were prospectively Cellscan tested and a clear-cut diagnostic distinction between malignant and non-malignant cases was apparent. These results strongly support the diagnostic value of such testing in suspected breast cancer patients in whom, in our study, 95% of the women with malignant breast tumour were also found positive by the Cellscan. No indication was found to support age influence on test performance.

However, in the population with benign tumours, the power of the Cellscan to rule out malignancy in the breast was 79.4%. In the control group, the Cellscan proved to be a specific test which distinguished 98.6% of tumour-free women.

Table 1. Characteristics of patients and correlation of the Cellscan results with the pathology

	No. of patients	No. of Cellscan positives (%)	No. of Cellscan negatives (%)
Healthy volunteers (control)	73	1 (1.4)	72 (98.6)
Benign tumours	63	13 (20.6)	50 (79.4)
Breast carcinomas	80	76 (95.0)	4 (5.0)
Staging			
I	27	26 (96.3)	1 (3.7)
II	30	28 (93.3)	2 (6.7)
III	21	20 (95.2)	1 (4.8)
IV	2	2 (100.0)	0 (0.0)

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