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Fluorescence Polarisation Changes in Lymphocyte Cytoplasm as a Diagnostic Test for Breast Carcinoma

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Lymphocytic cytoplasm from individuals with malignant disease, and from those without, differ in such a way as to be diagnostic both of malignancy generally and of specific types of cancer. Mitogenic stimulation of lymphocytes by phytohaemagglutinin (PHA) and antigenic stimulation by encephalitogenic factor (EF) and certain specific tumour-associated antigens, provokes changes in the structure of the cytoplasmic matrix (SCM) which are detectable upon fluorescence polarisation. The degree of change is quantifiable both by calculating the polarisation ratio (PR, polarisation before and after stimulation) and the relative ratio (RR_{SCM}, the ratio between the polarisation obtained after exposure to EF [P_{EF}] and to the polarisation measured after exposure to PHA [P_{PHA}]). A new tumour-associated antigen specific for breast cancer, CaBr, was tested for its diagnostic efficacy in comparison with that of EF, by prospectively testing blood samples from 138 consecutive women with suspicious breast masses. The previously known discriminatory power (sensitivity 60.7% and specificity 90.7%) of the polarisation-derived RR_{SCM} was reconfirmed. However, the RR'_{SCM} (the new ratio using CaBr instead of EF), was significantly more sensitive (77.4%; $P < 0.01$) and specific (94.4%) than the RR_{SCM} in detecting breast cancers. The polarisation changes in the cytoplasmic matrix after stimulation by CaBr alone suggest the best discriminatory power (sensitivity 90.5% and specificity 94.4%) between cancerous and non-cancerous patients.

Key words: breast cancer, fluorescence, polarisation, lymphocyte
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INTRODUCTION

INTRALYMPHOCYTIC PLASMA viscosity varies during a normal cell cycle [1], as well as in the presence of malignancy [2], and in response to specific antigenic stimulants, with consequent effects on the plasma membrane itself and its function in transmembranal transport kinetics [3] and other membrane-based enzymatic functions [4, 5]. The state of the lipid–protein gel transition [6], known as the structuredness of the cytoplasmic matrix (SCM), is physically measurable by spectrofluorometry using a fluorescein marker; the measured phenomenon is fluorescein fluorescence polarisation (FFP).

The specificity of SCM spectrofluorometric changes in lymphocytes in response to individual antigenic stimulants has been noted [2, 7, 8], and depends upon the presence or absence of malignancy in the cell donor. Incubation of lymphocytes from cancer-free subjects with phytohaemagglutinin (PHA) results in a decrease in the degree of fluorescence polarisation, while use of encephalitogenic factor (EF) or tumour cell line extracts does not. However, the lymphocytes of cancer patients, while unresponsive to PHA, manifest a decrease in fluorescence polarisation after incubation with antigenic substances consisting of either EF or tumourous extracts derived from the same type of malignancy from which the patient suffers [9].

The consistency of these SCM changes as measured by the FFP procedure constitute the basis of the SCM test for malignancy. The results are expressed by use of the polarisation ratio (PR) and the relative ratio for the SCM test. The PR is defined as:

$$PR = (P_0 - P_{Stimulant}) / P_0$$

where P_0 is the degree of polarisation before antigen stimulation and $P_{Stimulant}$ is the degree of polarisation after exposure to a specific antigen such as EF or CaBr (extract of breast cancer).

From the polarisation values measured after exposure to EF, PHA and CaBr, the relative ratio of SCM alteration attributable to each antigenic stimulant may be calculated according to the formula:

$$RR_{SCM} = P_{Stimulant} / P_{PHA}.$$

It has been empirically found that a value of $RR_{SCM} > 1$ militates against a malignancy, while an $RR_{SCM} < 1$ suggests it [9–11].

We aimed to compare the sensitivity and specificity of the previously developed P_{EF}-based RR_{SCM} with a new RR'_{SCM}, based on results obtained from CaBr, a mixture of extract of

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Table 1. Polarisation values ($P_{Stimulant}$) of lymphocytes from cancer-free and breast cancer patients after antigenic stimulation (with standard deviations and P values indicated)

Antigenic stimulant	$P_{Stimulant}$ in cancer-free patients	$P_{Stimulant}$ in breast cancer patients
PHA	0.149 ± 0.015	0.170 ± 0.023 $P < 0.0001$
EF	0.180 ± 0.013	0.166 ± 0.027 $P < 0.0002$
CaBr	0.182 ± 0.012	0.154 ± 0.014 $P < 0.00001$

three types of breast tumour tissue and one cell line, which was developed in 1988 for use as a specific provocative stimulant in the SCM-testing of lymphocytes for indications of breast cancer in the donor. This antigenic extract, CaBr, underwent limited retrospective examination with promising results [9]. We present the results of prospective SCM testing of a larger group of 138 consecutive female patients with undiagnosed breast masses.

The diagnostic implications of such a non-invasive test for both general and specific malignancy are potentially very far-reaching; the challenge, as for other diagnostic tools, is to bring the sensitivity and specificity of the test as close as possible to 100%.

PATIENTS AND METHODS

Lymphocytes for SCM testing were collected from 30 ml of peripheral blood drawn into heparinised polystyrene syringes from 138 consecutive women (aged 36–84 years) with palpable breast masses, referred to the Department of Oncology, Ichilov Hospital, Tel Aviv over a predetermined period of several months. All women subsequently underwent physical examination, mammography and biopsy. Eighty-four were found to have cancer while 54 were cancer-free.

The lymphocytes harvested from each patient were then prepared for SCM testing by separation into subgroups on a Ficoll–Triosil gradient solution as outlined by Cercek and Cercek [12] and modified in our laboratory [11]. The suspension of lymphocytes from each patient was divided into three 0.2-ml samples (5×10^6 lymphocytes/ml suspended in phosphate-buffered saline), each one for incubation (30 min at 37°C) with 20 μg of a separate antigenic stimulant. The final compositions of the three stimulants were 20 μg of reconstituted PHA

Table 2. Mean sensitivities and specificities of the SCM test parameters: PR and RR_{SCM} with 95% confidence intervals

Antigenic stimulant; PR and RR_{SCM}	Sensitivity	Specificity
PR_{PHA}	19.0% (10.6–27.4%)	96.4% (91.3–100%)
PR_{EF}	59.5% (49.0–70.0%)	90.7% (83.0–98.4%)
PR_{CaBr}	90.5% (84.2–96.8%)	94.4% (88.3–100%)
RR_{SCM} (EF:PHA)	60.7% (50.3–71.1%)	90.7% (83.0–98.4%)
RR'_{SCM} (CaBr:PHA)	77.4% (68.5–86.3%)	94.4% (88.3–100%)

PR, polarisation ratio; RR, relative ratio; SCM, structuredness of the cytoplasmic matrix; PHA, phytohaemagglutinin; EF, encephalitogenic factor; CaBr, tumour breast extract.

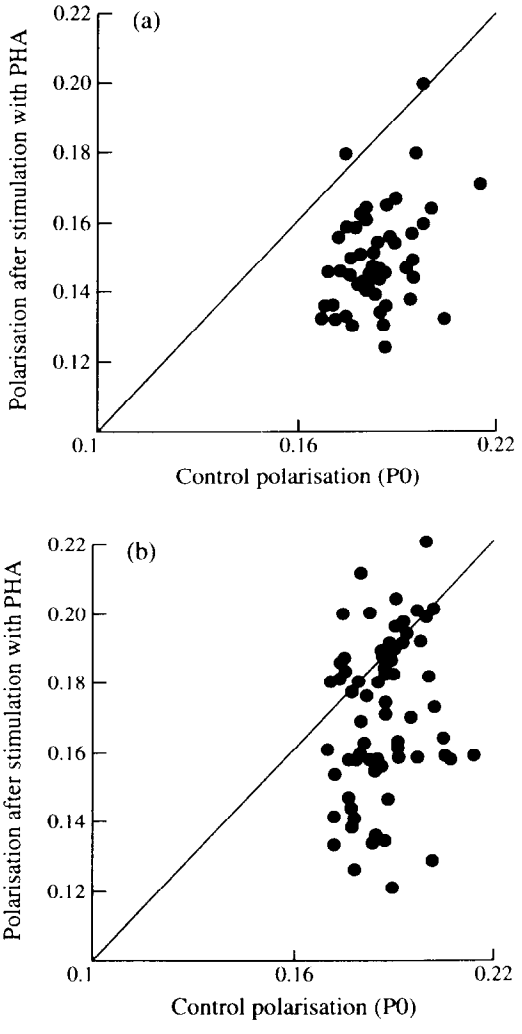

Figure 1. Patient response to phytohaemagluttinin (PHA) stimulation versus P_0. (a) Polarisation in women without breast cancer. (b) Polarisation in women with breast cancer.

(Wellcome, Dartford, Kent, U.K.); 20 μg of EF (Penninsula Laboratories, Belmont, California, U.S.A.); and 20 μg extract of CaBr. These concentrations had been previously empirically determined [9, 11].

CaBr, the antigenic breast extract under examination, was derived from a breast tissue cell line (T47D) with three cancers of differing histological types. In each case, pellets (1–3 g) of tumour or cell line tissue were thoroughly and multiply rinsed in phosphate-buffered saline, suspended in 5 volumes (v/v) of 3 M KCl (pH 7.4) and homogenised. After being stirred overnight at 4°C, the homogenate was centrifuged at 20000 *g* for 30 min at 4°C. The KCl was eluted by gel filtration through a Sephadex G-25 column and the remainder precipitated with 2 M (final concentration) ammonium sulphate for 1 h at 4°C, followed by centrifugation for 30 min at 20000 *g*, again at 4°C. The precipitate was suspended in doubly-distilled water and filtered through the Sephadex G-25 column to remove the ammonium sulphate. The eluted solution was then lyophilised and the power diluted in PBS at 10 mg/ml and stored at −90°C. The optimal concentration of the extracts solution were found empirically.

The FFP procedure was initiated by the addition of the

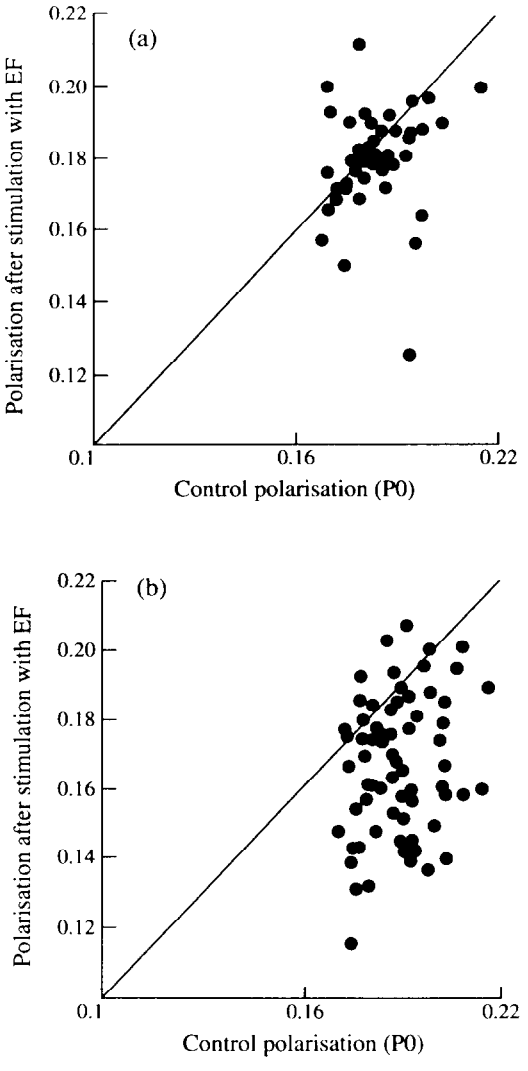

Figure 2. Patient response to encephalitogenic factor (EF) stimulation versus P_0. (a) Polarisation in women without breast cancer. (b) Polarisation in women with breast cancer.

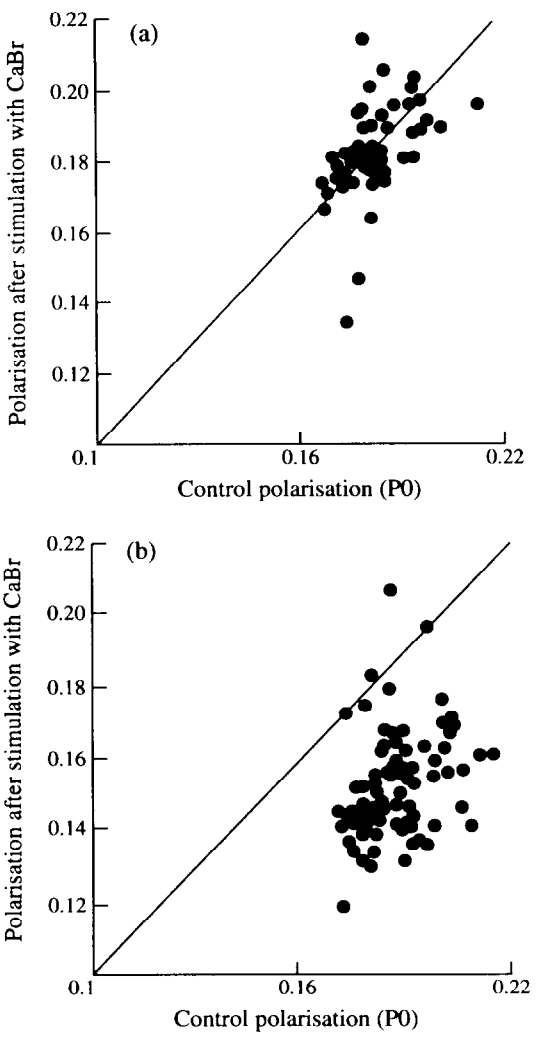

Figure 3. Patient response to extract of breast cancer (CaBr) stimulation versus P_0. (a) Polarisation in women without breast cancer. (b) Polarisation in women with breast cancer.

antigen-stimulated lymphocytes to 3.5 ml of the fluorescein diacetate (FDA) solution in order to promote the development of the marker, fluorescein. The polarisation of the emitted fluorescent light was then measured with a Perkin–Elmer (MPF-44) spectrofluorometer, allowing calculation of the polarisation (Table 1). The PR values were then used to derive estimates of sensitivity and specificity of the test for each of the various antigenic combinations.

The differences in these PR values were statistically significant ($P < 0.01$), allowing the discriminating power of the test results of the antigenic stimulants to be evaluated.

Statistical analysis

The confidence intervals for sensitivity and specificity were calculated in the standard manner for binominal variates. Comparison of central values for the different groups of subjects were made using Wilcoxon's non-parametric methodology. Comparison of the sensitivity values was effected by the McNemar test [13].

RESULTS

Of the 138 women investigated for breast masses, 84 (61%) proved to have a breast malignancy while the remaining 54 (39%) had benign breast conditions. In discriminating between these two groups, the three stimulants performed differently as indicated by their sensitivities (proportion of truly diseased who were diagnosed by the test) and specificities (proportion of healthy subjects so identified by the test) (Table 2).

The performance of PHA was highly specific; 96.3% of the non-cancerous patients responded with a significant ($P < 0.0001$) decrease in PR, suggesting their true cancer-free status (Table 2). However, 19% of the breast cancer group also responded to PHA stimulation (Figure 1), which constituted a group of PHA false negatives.

When EF stimulation was used, the SCM test sensitivity was 59.5%; 50 of the 84 breast carcinoma patients responded with the predicted decrease in the degree of fluorescence polarisation ($P < 0.0002$) (Figure 2). Five of the 54 cancer-free patients (9.3%) showed a falsely positive response when EF was used. In 114 patients (82.6%), both EF and CaBr antigens differentiated easily and gave compatible results for the two groups of patients. In 24 patients (17.4%), there was lack of correlation between

CaBr and EF results. Of these 24, 18 had breast carcinoma, 16 were detected by CaBr but missed by EF, and the other 2 were detected by EF but missed by CaBr. Of the 6 cancer-free patients who were assigned falsely positive diagnoses of cancer, 3 were due to CaBr and 3 to EF. CaBr antigen was the most sensitive in provoking an appropriate response; 90.5% (76 of 84 carcinoma patients) manifested decreases of PR ($P < 0.00001$) during the FFP procedure (Figure 3). 51 of 54 cancer-free patients were identified, giving a specificity of 94.4%.

DISCUSSION

The physical phenomenon of reactive structural change at the level of lymphocytic plasma in the presence of malignant disease now has an important diagnostic application in that lymphocytes from patients with malignant disease can be differentiated from those of cancer-free or at least non-malignant subjects [12, 14–17]. The efficacy of the non-invasive SCM test in detecting early cancer of the breast [9], stomach [18], urogenital organs [19], and colon and rectum [20] has been documented. The test parameters include PR and SCM relative ratios (RR_{SCM}) derived from the polarisation measured after exposure to a particular antigen relative to that obtained after exposure to PHA which is known not to affect the lymphocytic SCM of cancer patients. The RR_{SCM} is usually <1 in cancer patients.

In the present study of 138 women with suspected breast cancer, peripheral blood lymphocytes were prospectively SCM-tested and both polarisation and relative ratios were obtained; a clear-cut distinction between malignant and non-malignant cases was apparent after stimulation with PHA, EF and the breast tumour extract, CaBr (Table 1).

PHA proved to be a specific test which distinguished 96.3% of 54 cancer-free women. EF antigenic stimulation provoked SCM changes which identified 59.5% (50 of 84) breast cancer patients, making it the weaker discriminator of lymphocytes from diseased patients. Its performance in singling out cancer-free women (90.7%) was considerably more impressive. However, CaBR was more sensitive to breast cancer than EF and correctly confirmed the diagnosis in 90.5% (76/84) patients with the disease. In addition, its specificity (94.4%) nearly equalled that of PHA.

These results strongly suggest the diagnostic value in suspected breast cancer patients of stimulated SCM changes as measured by PR; in such a group the positive predictive value was 96.7% and the negative predictive value was 86.4%. However, in the population at large with a lower prevalence, the power to positively predict would markedly decline while negative predictive power would rise, thus limiting its use as a screening tool.

Our results confirm previous retrospective ones and help to establish the status of CaBr as a powerful, non-invasive, confirmatory diagnostic tool in breast cancer presenting as a palpable mass. We would hope to see prospective studies which would investigate its applicability in screening programmes for very early (preclinical) breast cancer.

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