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Validation of the SCM-test for the Diagnosis of Cancer

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Abstract—*The basic aspects of the SCM-test are summarized. A derivation of the fluorescence intensity vs time curve is given as well as that of the polarization as a function of time. These curves are found to be in excellent agreement with the experimental results. The test is applied to the lymphocytes of twenty-six healthy individuals and of thirty-three cancer patients. No false negative results were obtained. Seven cancer patients were found to be negative after various intervals from the removal of the tumour. The most important technical difficulties and pitfalls are discussed in order to facilitate the reproduction of the test by other researchers.*

INTRODUCTION

A RAPID test capable of diagnosing early malignant growths remains a key goal for cancer research. Thus the findings of Cercek *et al.* [1] on the changes of the fluorescence polarization in human lymphocytes after fluorochromasia of fluorescein [2] have attracted wide attention. According to this test the change in the degree of polarization in response to the mitogen phytohaemagglutinin (PHA) or to cancer basic protein (CaBP) and encephalitogenic factor (EF) indicates the absence or presence of a malignant disease in the blood donor.

When a well-defined group of lymphocytes from cancer patients are exposed to CaBP or EF the fluorescence polarization decreases on average by 20%. When exposed to PHA, however, there is no significant change in the fluorescence polarization (P value). In healthy individuals and in patients with non-malignant diseases the opposite occurs. An average decrease in the fluorescence polarization of 20% is observed after the lymphocytes have been stimulated with PHA, while no significant change occurs after stimulation with CaBP or EF. The result of the SCM test is best expressed by the magnitude of the parameter RR_{SCM}, which is defined as the ratio between the degree of polarization after stimulation with CaBP or EF and the degree of polarization after stimulation with PHA:

$$RR_{SCM} = \frac{P_{CaBP(EF)}}{P_{PHA}}.$$

Hence values of RR_{SCM} greater than unity are indicative for normals or patients with non-malignant diseases, while values of RR_{SCM} of less than unity indicate a state of malignancy.

Confirmation of the Cerceks' data and critical evaluation of their claims as to the sensitivity and efficiency of the test in the early detection of cancer have been slow to appear. Few groups have succeeded in repeating the test. Others have experienced varying degrees of technical difficulties and consequently the test has become controversial.

We have succeeded in confirming the validity of the SCM method in our laboratory, though so far for only a limited number of patients. The data will be given in the Results section. The successful reproduction of the test seems to depend on the solution of two major problems: (1) the separation of the proper subpopulation of lymphocytes of accurately defined density; and (2) the accurate measurement of the fluorescence polarization. May it suffice to note that in our earlier density measurements we neglected the weight of the air in the pycnometer and consequently achieved much poorer results.

Since the protocol of the test has been described exhaustively by the Cerceks [3] we will not repeat any of the details here. Several researchers, however, report polarization measurements which are in principle erroneous. In order to avoid such errors we give in the following a proof for the proper behaviour of a polarization curve. Although a realistic experimental polarization vs time curve is certainly the outcome of numerous parameters, some probably as yet unknown, results which

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deviate essentially from this curve should be suspected of being influenced by some artifact.

THE POLARIZATION CURVE

Intensity

When the properly prepared lymphocytes are introduced into a solution of fluorescein diacetate (FDA), which is a non-fluorescent solute, the molecules of FDA diffuse into the cell, where they become transformed into fluorescent fluorescein. The polarization of this fluorescence when excited by polarized light is measured as a function of time shortly after introduction of the cells into the FDA solution. Due to the small number of cells the concentration of FDA in the solution is assumed. (Under the conditions of the test the fraction of FDA converted into fluorescein is less than 1%.) Since the cell walls are not impermeable to fluorescein part of the fluorescein which accumulates in the cells will leak out of them. Since the viscosity of the solvent is very low the polarization of the fluorescence of the fluorescein which has leaked out of the cells will be negligible. This fluorescence will represent a serious background which increases with time and affects the results considerably. In order to correct the results for this effect the cells are sucked out of the suspension and the remaining background fluorescence is measured for the two positions of the polarization analyser.

We now describe these effects in a quantitative manner.

Let F_1 be the number of (fluorescing) fluorescein molecules in the lymphocytes and F_A the number of (non-fluorescent) fluorescein diacetate molecules in the solvent.

Then

$$\frac{dF_1}{dt} = \alpha F_A - \beta F_1,$$

where α is the rate constant for the formation of F_1 by the lymphocytes (which is mainly determined by the diffusion of F_A into the lymphocytes) and β is the rate of diffusion (leaking) of F_1 out of the lymphocytes. (Back-diffusion of fluorescein into the lymphocytes is negligible [2].) If F_2 denotes the number of fluorescein molecules which have leaked out of the lymphocytes into the solvent (PBS), then obviously

$$\frac{dF_2}{dt} = \beta F_1.$$

Assuming $F_1 = 0$ and $F_2 = 0$ for $t = 0$, we obtain

$$F_1(t) = \frac{\alpha}{\beta} F_A(1 - e^{-\beta t})$$

$$F_2(t) = \frac{\alpha}{\beta} F_A(\beta t + e^{-\beta t} - 1).$$

The overall number of fluorescent molecules

$$F_{\text{total}}(t) = F_1(t) + F_2(t) = \alpha F_A(t)$$

is thus linearly increasing with time.

The functions $F_1(t)$, $F_2(t)$ and $F_{\text{total}}(t)$ are graphically presented in Fig. 1. From this figure it is immediately seen that the sooner the measurement is performed after introduction of the lymphocytes into the FDA solution the more favourable the ratio of signal ($F_1(t)$) to background ($F_2(t)$). This behaviour has been observed in a vast number of experiments and it is the precondition for any further experimentation on the SCM test.

Polarization

Now let us proceed with the description of the polarization as a function of time. Since the fluorescence of F_2 is unpolarized its intensity will be equally distributed among all directions of polarization, i.e. one-third for each direction. For the evaluation of $F_1(t)$ we refer to Fig. 2. If θ describes the angle subtended by the molecular oscillator A of fluorescein with the axis of polarization of the exciting radiation, which is assumed to be parallel to the Z axis (the absorption and emission oscillators are assumed to coincide), and ϕ is the angle between the projection A' of the oscillator in the xy plane and the y axis, which is the direction of detection, then the intensities $I_\parallel$ and $I_\perp$ of the parallel and normal components of the measured fluorescence will be

$$I_\parallel = I_0 \gamma \left(F_1(t) \overline{\cos^2\theta} + \frac{F_2(t)}{3} \right) \qquad (1)$$

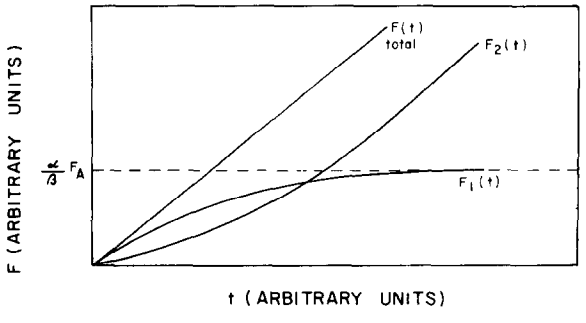

Fig. 1. The fluorescence intensity of fluorescein in the lymphocytes $F_1(t)$, in the solution $F_2(t)$ and the overall fluorescence intensity of the cell suspension $F(t)$ as functions of time.

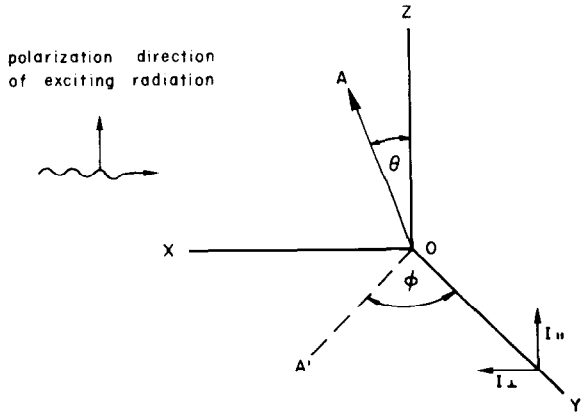

Fig. 2. Directions of axes, molecular dipole and polarization vector for the derivation of equations (1) *and* (2).

and

$$I_\perp = I_0\gamma\left(F_1(t)\,\overline{\sin^2\theta}\;\overline{\sin^2\phi} + \frac{F_2(t)}{3}\right), \qquad (2)$$

where I_0 is the intensity of the exciting radiation (in the Z direction), γ is the quantum yield of fluorescein (assumed to be equal for the molecules in the lymphocyte and those in the solution) and the bars above the trigonometric functions indicate their average values. Since the distribution of excited dipoles is symmetrical around the Z axis, $\overline{\sin^2\phi} = 1/2$.

The measured degree of polarization P is

$$\begin{aligned} P(t) &= \frac{I_\parallel(t) - I_\perp(t)}{I_\parallel(t) + I_\perp(t)} \\ &= \frac{F_1(\overline{\cos^2\theta} - (1/2)\,\overline{\sin^2\theta})}{F_1(\overline{\cos^2\theta} + (1/2)\,\overline{\sin^2\theta}) + (2/3)F_2} \\ &= \frac{3(\overline{\cos^2\theta} - (1/3))}{(\overline{\cos^2\theta} + 1 + (4/3)(F_2/F_1))}. \end{aligned}$$

Since $(F_2/F_1) = \beta t/(1 - e^{-\beta t}) - 1$ increases with time the measured degree of polarization decreases with time. On a record of $I_\parallel$ and $I_\perp$ as functions of time this fact will be expressed by an upward bending of the $I_\perp$ curve and a downward bending of the $I_\parallel$ curve with increasing time. This is seen by the explicit expression of $I_\perp$ and $I_\parallel$ as functions of time:

$$\begin{aligned} I_\perp(t) &= I_0\gamma\left(\frac{F_1}{2}\,\overline{\sin^2\theta} + \frac{F_2}{3}\right) \\ &= I_0\gamma\left[\frac{\alpha}{\beta}\frac{F_A}{2}(1 - e^{-\beta t})\,\overline{\sin^2\theta}\right. \\ &\quad \left. + \frac{1}{3}\frac{\alpha}{\beta}F_A(\beta t + e^{-\beta t} - 1)\right] \\ &= \frac{1}{2}I_0\gamma\frac{\alpha}{\beta}F_A\left[(\overline{\sin^2\theta} - \frac{2}{3})\left(1 - e^{-\beta t}\right) + \frac{2}{3}\beta t\right]. \end{aligned}$$

Since P is positive the average value of θ is less than 45° and hence

$$\left(\overline{\sin^2\theta} - \frac{2}{3}\right) < 0$$

$$\frac{d^2 I_\perp(t)}{dt^2} = \text{const.} \times e^{-\beta t}$$

is positive everywhere, which describes an upward bended curve.

$$\begin{aligned} I_\parallel(t) &= I_0\gamma\left(F_1\overline{\cos^2\theta} + \frac{F_2}{3}\right) \\ &= I_0\gamma\frac{\alpha}{\beta}F_A\left[\left(\overline{\cos^2\theta} - \frac{1}{3}\right)(1 - e^{-\beta t}) + \frac{1}{3}\beta t\right], \end{aligned}$$

with

$$\left(\overline{\cos^2\theta} - \frac{1}{3}\right) > 0$$

for the same reason as above. Hence

$$\frac{d^2 I_\parallel(T)}{dt^2} = -\text{const.} \times e^{-\beta t}$$

is negative everywhere, which means a downward bending of the curve. A representative graph of an actual measurement is given in Fig. 3. The absolute values of the intensities are strongly determined by the reflection properties of the analysing monochromator. This technical feature causes the normal component to appear stronger than the parallel component and is of no intrinsic significance.

METHOD

Modified Dulbeco PBS was prepared with the following modifications: osmolality: 330 mosm/kg, Ca^{2+} concentration: 2 mM, FDA (Riedel-de Haen Ag. Seelze-Hanover), PHA (Wellcome Ltd.), EF (Beckman Co. preparation prior to 1981), acetic acid (Frutaron), Ficoll 400 (Pharmacia AB), Triosil (Nyegaard and Co. AS, Oslo).

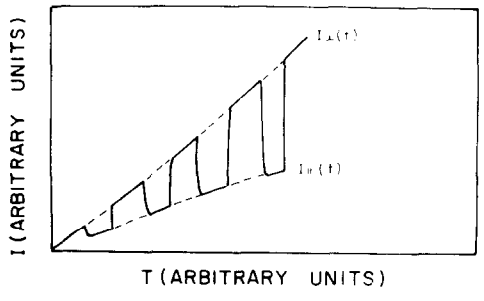

Fig. 3. The intensity of the parallel and normally polarized component of the fluorescence.

PHA was reconstituted in 5 ml of double-distilled water and further diluted ten times. For stimulation 0.1 ml of this solution was added to 1 ml of cell suspension. EF was reconstituted in 2 ml of double-distilled water. For stimulation 0.1 ml of the solution was added to 1 ml of cell suspension.

FDA solution (0.6 μM) was prepared as follows: 50 mg of FDA was dissolved in 5 ml of acetic acid by gently warming and shaking. Ten microlitres of this solution was added to 100 ml of the modified PBS, to which 0.37 g of $Na_2HPO_4 \cdot 12\,H_2O$ had been added (for correction of the pH). Twenty-five millilitres of this solution was further diluted in 75 ml of the modified PBS. The cell suspension (0.2 ml; 5×10^6 cells per ml) was introduced into 3 ml of the FDA solution.

The gradient solution was prepared in the following way: a solution of 23 g Ficoll in 250 ml water was added to a solution of 51 ml Triosil in 51.3 ml water at a ratio of 12:5. The density of the gradient solution was determined at a given temperature by pycnometry. The pycnometer was calibrated with double-distilled water, correcting for the weight of air. The final pH of the gradient solution was between 5.5 and 6.2. The temperature of lymphocyte separation was determined by the respective relationship between temperature density and osmolality [4]. The osmolality varied between 310 and 325 mosm/kg.

The centrifuge (Runne—model 101E-K-Ti) separation time was 20 min at 550 *g* (at the radius of the blood and gradient interface) at the selected temperature, which was kept constant within 0.1°C.

After the separation, two cell layers were obtained in most cases. These were separated by a distance of 1 mm. The upper layer was carefully removed and used for the SCM test. In those cases in which only one layer was observed, this was taken, leaving the lowest stratum of cells in the solution.

The fluorimetric measurements were performed on a Perkin Elmer MPF-44 spectrofluorimeter. The wavelength of excitation of the fluorescein fluorescence was 470 nm, with a bandwidth of 20 nm; in order to reduce the stray light an interference filter (Balzers) for 468 nm was further interposed in the excitation beam.

The fluorescence was monitored at 510 nm, with a bandwidth of 6 nm. The G-factor of the detection grating was 0.551.

For the background determination the cells were filtered out of the solution by a 0.22-μm pore-size filter (Tamar).

RESULTS

The clinical observations on the cancer patients were all made at the Sheba Hospital at Tel-Hashomer, Israel. The blood samples were taken from a random population of the oncological outpatient clinic there. Only after performance of the SCM-test were the results compared with the clinical records.

Table 1 shows the results obtained for 26 healthy donors and 33 cancer patients. Each result is the average of several (3–6) runs. Typical S.D. values for these averages are less than 4%. A few samples have been tested by PHA activation only since at the time of these tests we have not yet obtained EF. In the table P_0 denotes the degree of polarization prior to stimulation, and P_{PHA} and P_{EF} denote the degree of polarization after stimulation of the lymphocytes with PHA or EF respectively. RR_{SCM} is the ratio of P_{EF}/P_{PHA}.

DISCUSSION

Seven out of the 33 cancer patients had RR_{SCM} values greater than unity. Patients 43, 44, 49, 50, 51 and 53 have been free from any clinical symptoms for at least one year and are under routine supervision, receiving no chemotherapy, radiation or other treatment; patient 37 was tested one month after removal of tumour. The SCM-test serves thus as a follow-up procedure for the immunological status of the patients. The limited number of cases reported here includes no false positives nor false negatives.

The exact values of P_0, P_{PHA} and P_{EF} are not very meaningful since slight changes in the composition of the PBS, which may vary from batch to batch, give slight changes in the absolute values of the polarization. Only the ratio of these values has diagnostic significance. Still, very low P values of the control sample (P_0) usually indicate a faulty treatment of the lymphocytes at one of the steps of the test or some failure in the measuring system. Such measurements should therefore be discarded. We have also not studied the relationship between the absolute value of RR_{SCM} (except for the inequality $< \text{or} > 1$) and the degree of the disease, though there are indications that such a relationship exists and may be of diagnostic value. Much more work, however, is needed to make this a meaningful parameter.

There are many ways by which a successful execution of the test can be averted and almost any deviation from the strict adherence to the protocol prescribed by the Cerceks is a sure means for failure. It may therefore be of some

Table 1. Degree of polarization before (P_0) and after stimulation (P_{PHA}, P_{EF}) and RR_{SCM} values

	Donor	P_0	P_{PHA}	P_{EF}	RR_{SCM}
Healthy donors					
1		0.235	0.181		
2		0.198	0.156		
3		0.211	0.157		
4		0.201	0.156		
5		0.195	0.154		
6		0.210	0.160		
7		0.200	0.149		
8		0.204	0.155	0.213	1.37
9		0.189	0.144	0.190	1.32
10		0.203	0.169	0.203	1.20
11		0.196	0.144	0.227	1.58
12		0.199	0.157	0.188	1.20
13		0.198	0.164	0.198	1.21
14		0.200	0.156	0.200	1.28
15		0.187	0.141	0.189	1.34
16		0.185	0.154	0.192	1.25
17		0.182	0.147	0.193	1.31
18		0.198	0.150	0.193	1.29
19		0.212	0.157	0.197	1.25
20		0.189	0.162	0.206	1.27
21		0.194	0.153	0.195	1.27
22		0.192	0.176	0.226	1.28
23		0.193	0.153	0.179	1.17
24		0.186	0.155	0.194	1.25
25		0.185	0.165	0.190	1.15
26		0.204	0.159	0.194	1.22
Cancer patients					
27	Carcinoma of breast	0.220	0.215		
28	Carcinoma of kidney	0.210	0.212		
29	Carcinoma of lung	0.210	0.205		
30	Lymphosarcoma	0.223	0.229		
31	Carcinoma of breast	0.209	0.205		
32	Malignant melanoma	0.193	0.200	0.168	0.84
33	Carcinoma of bladder	0.191	0.189	0.157	0.83
34	Adenocarcinoma of endometrium	0.182	0.185	0.158	0.85
35	Carcinoma of colon	0.189	0.186	0.160	0.86
36	Testicular seminoma	0.213	0.218	0.172	0.79
37	Adenocarcinoma of colon	0.196	0.183	0.205	1.12*
38	Carcinoma of lung	0.192	0.185	0.156	0.84
39	Gastric carcinoma	0.185	0.187	0.158	0.84
40	Carcinoma of breast	0.177	0.166	0.147	0.88
41	Carcinoma of ovary	0.177	0.182	0.127	0.70
42	Adenocarcinoma of colon	0.193	0.188	0.133	0.71
43	Adenocarcinoma of stomach	0.200	0.134	0.163	1.22*
44	Testicular carcinoma	0.200	0.140	0.213	1.52*
45	Malignant melanoma	0.199	0.194	0.142	0.73
46	Carcinoma of breast	0.195	0.190	0.151	0.79
47	Carcinoma of endometrium	0.204	0.206	0.185	0.90
48	Carcinoma of breast	0.200	0.186	0.155	0.83
49	Malignant melanoma	0.195	0.155	0.189	1.22*
50	Intraductal carcinoma of breast	0.184	0.148	0.189	1.28*
51	Adenocarconoma of vecto sigmoid	0.182	0.165	0.185	1.12*
52	Carcinoma of ovary	0.197	0.199	0.143	0.72
53	Malignant melanoma	0.191	0.166	0.186	1.12*
54	Carcinoma of breast	0.190	0.190	0.139	0.73
55	Carcinoma of colon	0.195	0.204	0.145	0.71
56	Carcinoma of breast	0.192	0.184	0.160	0.87
57	Carcinoma of breast	0.187	0.195	0.153	0.78
58	Carcinoma of colon	0.195	0.221	0.139	0.63
59	Carcinoma of breast	0.213	0.211	0.143	0.68

*No clinical symptoms after removal of tumour.

value for future experimenters to underline a few points which we found particularly important.

1. The importance of choosing the proper subpopulation of lymphocytes with regard to density cannot be over-emphasized. We mentioned one possible pitfall in the Introduction section. A variation of 0.1% of the density requires a change of 3°C in the temperature at which the separation has to be performed. Therefore, we adjusted the temperature of separation for every new batch of gradient solution which we prepared and controlled the proper temperature within 0.3°C.

2. The overall yield of pure lymphocytes after isolation should be 90% and the P value of the control (i.e. before stimulation) should not be less than 0.18. Whenever a lower value was obtained we checked the osmolality, pH and Ca^{2+} concentration of the (PBS) FDA solution and the temperature of the cuvette holder. If after correction of these parameters the P value is still below 0.18 it may safely be assumed that the wrong lymphocyte population is being measured and the test should be discontinued. (In several cases we also applied the second criterion for the proper lymphocyte population, which is a ratio > 1.3 between the degrees of polarization at 510 and 515 nm.) Once the proper routine is developed such occurrences will be rare. Out of the 59 tests reported here only 2 had to be interrupted and repeated by application of this criterion.

Some authors [5] reported a decrease in the degree of polarization with increasing concentration of fluorescein in the lymphocytes. We have not experienced this effect under the conditions of our experiments. Extending the recording time from 3 to 12 min (when there were less lymphocytes in the cuvette) did not cause any change in the degree of polarization, though obviously the accumulated concentration of fluorescein within the lymphocytes was considerably higher in the longer measuring interval. Any change in the degree of polarization with increasing concentration of fluorescein which might have occurred was within the 3% accuracy limits of the experiment.

Our experiments are also at variance with other findings of these authors [5]. For example, they found an increase in the rate of fluorochromasia upon stimulation of the lymphocytes with PHA (from which fact they tended to interpret the decrease in the degree of polarization as a consequence of the well-known phenomenon of concentration depolarization), whereas our experiments show a decrease in fluorochromasia upon PHA stimulation.

4. In order to evaluate the background fluorescence the lymphocytes are rather speedily filtered out of the suspension and the fluorescence of the remaining solution is measured. We did not observe any difference in the background reading when we changed from a manually operated vacuum pump to an automatic constant pressure–constant time filtering device. Some authors [6] claim that this filtering process may introduce errors into the background reading.

5. Within an interval of 24 hr we have not found any change in the P values with the time which passes between the extraction of the blood from the donor and the separation of the lymphocytes when the blood is kept at room temperature. There was also no impairment of the test when the lymphocytes were separated on one day, kept in PBS at low temperatures and the rest of the test performed the next day. No change in the viability of the cells occurred. There was no difference between the P values of the controls when they were measured on both days and neither was there a change in the P values of the PHA-stimulated lymphocytes. The P values of the EF-stimulated lymphocytes showed, however, a decrease of about 10% as a result of this procedure, which consequently showed in the RR_{SCM} values. However, we have very recently been made aware (Cercek and Cercek, private communication) that for certain auto-immune diseases this procedure may reduce or alleviate the effect of PHA stimulation of the P value, so one should be cautious in its application.

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