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Changes in SCM-Responses of Lymphocytes in Mice after Implantation with Ehrlich Ascites Cells*

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Abstract—*BDF-1 mice implanted for various times and with different numbers of Ehrlich ascites cells were used as a model system to find out how early in the development of neoplasia the SCM-responding subpopulation of lymphocytes can detect cancer (SCM-test), as assessed by changes in intracellular fluorescein polarization in response to stimulation with phytohaemagglutinin and ascites cell extract (ACE). One hour after implantation of* 7.5×10^5 *ascites cells SCM-responses to ACE were induced and PHA responses abrogated. At the threshold number of* 3.5×10^5 *ascites cells per animal SCM-responses to both ACE and PHA were observed. Ascites cells killed by radiation did not induce changes in SCM-responses. Extrapolation of these data on the basis of body weight ratio indicates that a human tumour load of less than* 10^9 *cancer cells might be detectable in the SCM-test.*

INTRODUCTION

IN THE development of any cancer screening test it is important to establish how early the disease can be detected. The 'SCM-test', which is based on the responses of lymphocytes to mitogens and tumour proteins as detected by structural changes in mitochondria by intracellular fluorescein fluorescence polarization [1], was found to be positive in clinically early stages of the disease, e.g., in carcinomas *in situ* of the cervix [2] and in the early, pre-invasive stages of stomach cancer [3]. However, these apparently localised clinically detectable primary tumours can already be accompanied by occult metastases. The information on how early in the subclinical stage of the disease the SCM-responding subpopulation of lymphocytes starts to respond to cancer cell extracts and ceases to respond to phytohaemagglutinin cannot be deduced from the SCM-results on cancer patients. We have, therefore, used as a model system mice implanted with Ehrlich ascites tumour cells. The relationship between the number of ascites cells implanted and the time required to obtain SCM-responses in lymphocytes typical for cancer conditions [2–5] and mechanisms involved in the induction of SCM-responses were studied.

MATERIALS AND METHODS

Biological material

BDF-1, 7–8 weeks old male mice and in part of the experiments Nude 8 weeks old male mice (OLAC, 1976, Ltd., Shaws Farm, Blackthorn, Bicester, U.K.) were used. A well established line of Ehrlich ascites cells (originally obtained from the Mount Vernon Hospital, London) which in the Paterson Laboratories is routinely passaged in BDF-1 mice, was used for implantations and the production of ascites cell extracts (ACE).

Implantation procedures

Ascites cells were collected from the peritoneal fluid of tumour-bearing mice. The cells were washed three times with phosphate buffered saline (PBS) and re-suspended in PBS. These cell suspensions contained about 80% of ascites cells. Mice were injected intraperitoneally (i.p.) with 0.2 ml of cell suspensions containing the required ascites cell numbers. The control animals were injected with 0.2 ml of PBS only. For implantations of dead cells, washed ascites cells suspended in PBS were irradiated with a dose of 10 Krads

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delivered at the rate of 505 rads/min using a Cs-137 γ-ray source. To remove the radiolytic products in the PBS before implantation cells were washed twice and resuspended in PBS at the concentration of 10^6 cells/ml of which 0.2 ml were injected per mouse. In part of the experiments mice were injected with 0.2 ml of the ascites cell-free peritoneal fluid of tumour-bearing mice. Cells were removed by centrifugation of the ascitic fluid at 1500 ***g*** for 30 min. The cell-free supernatants were used for i.p. injections.

Seven to ten mice were used for each implantation and the same number as controls.

Preparation of ascites cell extracts (ACE)

Ascites cells from tumour-bearing mice were washed twice with PBS and suspended in PBS at about 10^6 cells/ml. The cell suspension was incubated at 37°C for 2–3 hr, centrifuged and the supernatant filtered through a Millipore filter (0.22 μm pore size). The filtrate contained 1.8–2.5 mg of protein per ml of extract, as determined by the Lowry method [6]. For stimulation of lymphocytes 0.1 ml of the ACE was added to 1.0 ml of the lymphocyte suspension (about 10^6 cells/ml) and incubated for 40–60 min at 37°C.

Isolation of SCM-responding lymphocytes

Blood was collected from the veins in the axillary region of anaesthetised mice using a pasteur pipette which was repetitively rinsed during collection with a heparin solution (100 i.u./ml). The blood from 7-10 treated or control animals was pooled into 20 ml McCartney vials rinsed with the same heparin solution. About 8 ml of blood was collected from 7–10 animals and was diluted with an equal volume of PBS. The diluted blood was divided into two McCartney's vials each containing 100 mg of carbonyl iron powder (8365 w, 99.5% iron powder, Koch-Light Ltd.). The samples were rotated at 30 rev/min for 30 min at 37°C and then placed on a magnet for 10 min. Before layering, both the blood and the density solution were equilibrated to the same temperature in a thermostatically controlled aluminium block. The SCM-responding lymphocytes were isolated by centrifugation of the blood at 1100 ***g*** for 20 min on a Histopaque density solution (batch 98C 6147, Pharmacia AB) at 10.9°C, as calculated from the osmolality and density of the Histopaque batch used [7]. The temperature of the blood and of the density solution before and during centrifugation was controlled to within ± 0.2°C. To prevent mixing of the lymphocyte layers by thermal diffusion currents the gradients were kept at 10.9°C until the collection of the SCM-responders. Only the density specific cells which separated within a 2 mm band above the visible interphase were collected, avoiding any cells which separated inside the gradient. The cells were washed twice with saline and once with PBS, resuspended in PBS at a concentration of about 6×10^5 cells/ml and kept in an incubator at 37°C [2, 5]. The differential counts showed that the cell suspensions contained over 95% pure lymphocytes. The largest contaminants were erythrocytes and negligible amounts of granulocytes. The lymphocytes consisted of about 87% of T-cells and about 13% of B-cells as determined by immunofluorescence using FITC-labelled rabbit anti-mouse θ antigen (Bionetics Ltd., Kensington, Maryland, U.S.A.) and rabbit anti-mouse IgG (Miles Lab., Stoke Poges, Slough, U.K.), respectively.

Stimulation of lymphocytes

Aliquots of lymphocyte suspension were incubated for 40–60 min at 37°C with either the reconstituted and ten times diluted reagent grade PHA (Gibco, Europe Ltd., Uxbridge, U.K.) or with the ascites cell extract (ACE). PHA and ACE were dosed at 0.1 ml/1 ml of cell suspensions.

Measurements of the intracellular fluorescein fluorescence polarization (SCM-technique)

The degree of fluorescein fluorescence polarization P of fluorescein molecules in lymphocytes was measured following procedures described previously [2, 5]. The technique is based on the excitation of fluorescein molecules, produced by intracellular enzymatic hydrolysis of the nonfluorescing fluoresceindiacetate (FDA), with polarized light and measurement of the degree of polarization of the emitted fluorescence. Aliquots of cell suspensions were injected at concentrations of not more than 3×10^5 cells/ml in 3 ml of 0.7×10^{-6} M FDA in complete PBS containing calcium and magnesium [2, 8]. The pH of the substrate was 7.45 ± 0.02 and the osmolality 0.330 ± 0.005 osmol/kg. This suspension was rapidly transferred into a 1-cm cuvette and put into the thermostated cuvette holder of the Perkin–Elmer MPF-4 fluorescence spectrophotometer (Perkin–Elmer Corp.). Polaroid type HNPB polarizers (Polaroid Corp.), which under our experimental conditions did

not exhibit any intrinsic fluorescence and transmitted on crossing <0.5% of light, were used. The instrument was equipped with an automatic emission-polarizer changer. To improve the resolution of the exciting and emitted light by monochromators and to eliminate stray light artifacts a primary blue FITC filter (Barr & Stroud Ltd., Glasgow, Scotland) was mounted between the excitation monochromator and polarizer as well as a GG495 cut-off filter (Barr & Stroud Ltd.) between the cuvette and the emission polarizer. A 2-fold increase in the intensity of the vertically polarized light was achieved by inserting a polaroid type HNCP 37 half-wavelength plate (Polaroid Corp.) between the FITC filter and the excitation monochromator oriented with its optical axis at an angle of 45° to the vertical position. Measurements were made at 27°C. The excitation monochromator was set at 470 nm (slit-width 20 nm) and the emission monochromator at 510 nm (slit-width 10 nm). Details of the experimental procedures and calculations of the intracellular fluorescein fluorescence polarization values P were the same as described before [2, 5].

RESULTS AND DISCUSSION

The intracellular fluorescein fluorescence polarization value P of SCM-responding lymphocytes from control and tumour bearing mice is the same, i.e. $P=0.210\pm0.005$ (maximal deviation). On stimulation with PHA lymphocytes of the controls responded with a 25–30% decrease in P-values, but did not respond to stimulation with ACE. The resulting SCM-response ratios, RR_{SCM}, (P_{ACE}/P_{PHA}) were between 1.4 and 1.5 (Table 1) similar to those reported for healthy human donors and patients with non-malignant diseases [2–5, 9–12]. The results in Table 1 show that already 1 hr after i.p. implantation of 7.5×10^5 ascites cells per mouse the SCM-responding lymphocytes responded to ACE and ceased to respond to PHA stimulation. The RR_{SCM} changed after 1 hr of implantation from 1.5 in controls to 0.78 in mice implanted with ascites cells, i.e., to an RR_{SCM}-value similar to that observed in patients with cancer [2–5, 9–12]. The number of ascites cells per animal required to induce changes in the SCM-responses of lymphocytes 2 hr after implantation is shown in Table 2. There is a progressive decrease in PHA-responses and an increase in SCM-responses to ACE as the number of implanted ascites cells is increased

Table 1. SCM-response to PHA and ascites cell extracts (ACE) at different times after implantation of 7.5×10^5 *ascites cells per animal*

Time after implantation	Polarization as % of control after stimulation of lymphocytes with: PHA	ACE	RR_{SCM}
0—control	67.0±4.0	100.0±1.5	1.50
1 hr	99.0±2.0	77.0±2.0	0.78
2 hr	99.4±1.5	75.0±2.0	0.76
48 hr	101.0±2.0	69.0±3.0	0.68
7 days	101.0±2.0	66.0±3.0	0.65

Each result is the mean value of three independent experiments.

Table 2. SCM-responses to PHA and ascites cell extracts 2 hr *after implantation of different numbers of ascites cells*

Number of ascites cells implanted per animal	Polarization as % of controls after stimulation of lymphocytes with: PHA	ACE	RR_{SCM}
7.4×10^4	70.0±2.0	100.0±1.5	1.43
3.5×10^5	88.0±1.5	80.0±2.0	0.91
6.5×10^5	97.0±2.0	74.0±1.5	0.76
7.5×10^5	99.4±1.5	75.0±2.0	0.75
8.5×10^5	100.0±2.0	74.0±2.5	0.74

Each result is the mean value of three independent experiments.

from 7.4×10^4 to 6.5×10^5 cells per animal, resulting in a decrease in the RR_{SCM}-value from 1.43 to 0.76. At 3.5×10^5 cells per animal SCM-responses to both ACE and PHA were detected, although responses to PHA were smaller than those observed in lymphocytes from control animals. This type of SCM-response was observed in human donors with pre-malignant diseases and in some cases histologically diagnosed as displasia cervix uteri or benign breast conditions [2]. Extrapolation of results from mice to the human on the basis of the body weight ratio indicates that a human tumour load of less than 10^9 cancer cells might be detectable. Assuming that this extrapolation is valid, the SCM-test could be positive already at the level of subclinical disease [13].

The presence of actively metabolizing ascites cells appears to be required for the induction of SCM-responses to ACE and abrogation of PHA responses. When the ascites cells irradiated with 10 Krad of γ-radiation or the cell-free peritoneal fluid from

tumour-bearing mice were injected into animals no response to ACE was induced and the lymphocytes responded normally to PHA stimulation.

The observation that already within 1 hr after implantation of ascites cells (Table 1) the SCM-responding lymphocytes exhibit responses to ACE suggested that ACE sensitizing and PHA response abrogating molecules could appear in the blood of tumour-bearing mice. To verify this hypothesis the SCM-responding lymphocytes from control animals were incubated for 2 hr at 37°C in the cell-free blood plasma from tumour-bearing mice. The lymphocytes were then washed twice with saline and once with PBS and resuspended in PBS. In these lymphocytes the SCM-responses to PHA were diminished by half and a 40–50% response to ACE stimulation was observed. The RR_{SCM}-values changed from 1.5 to 0.72 after incubation of lymphocytes in the plasma of tumour-bearing mice. The SCM-response-modifying molecules in the blood of tumour bearing mice can be inactivated by heating the plasma for 15 min at 56°C. The heat inactivated plasma no longer induces responses to ACE and abrogates or diminishes PHA responses in lymphocytes of control animals. Further studies to elucidate the nature of these plasma factors are in progress.

The density-specific SCM-responding subpopulation of lymphocytes consists of about 87% of T-type cells and about 13% of B-type cells. These fractions of T- and B-cells are similar to those reported in SCM-responding populations of lymphocytes isolated from human blood [7, 11, 14] and are in agreement with the observation that the SCM-responding cells are of the T-cell type [11]. To ascertain that the presence of T-cells is required for the induction of SCM-responses in lymphocytes to ACE, athymic Nude mice were implanted with ascites cells and the SCM-responses of lymphocytes were measured. In contrast to BDF-1 mice the density-specific lymphocytes from Nude mice gave no SCM-responses to ACE. Furthermore, lymphocytes from control Nude mice did not exhibit any SCM-responses to PHA; thus corroborating that the SCM-responding lymphocytes are of the T-cell type.

In conclusion, the above results show that already within 1 hr after implantation of 7.5×10^5 ascites cells into the animals the SCM-responding lymphocytes become sensitized to ACE and the SCM-responses to PHA are abrogated. The results also indicate that the same subpopulation of density-specific T-lymphocytes either responds to PHA in controls, or to ACE in tumour-bearing animals. Induction of SCM-responses to ACE and the concomitant abrogation of PHA responses seems to be caused by SCM-response-modifying proteins which appear in the blood plasma of mice implanted with ascites cells.

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