Cell-surface radioiodination with the sparingly soluble catalyst Iodogen

Differences between the dividing and non-dividing populations of rodent thymocytes

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The surface proteins of dividing and non-dividing subpopulations of rat and mouse thymocytes have been labelled by using a new method of radioiodination. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and autoradiography of the labelled proteins shows distinct differences in labelling between the mouse and rat cells and also, in the case of the rat, between the dividing and non-dividing populations.

An important difference between normal and tumour cells is that tumour cells apparently lack the ability to switch off cell division in response to the appropriate stimuli (Farber, 1973; Stoker, 1972). Furthermore, it has been widely postulated that the cell surface may be involved in the control of cell division (Pardee, 1971; Burger, 1971). Much work has therefore been carried out to compare the surface of normal cells with their malignantly transformed counterparts (Hynes, 1976; Nicolson, 1976). Many differences in surface-membrane composition and ultrastructure have been found between the two cell types, but these have shed little light on the neoplastic process. There have also been many studies on the events that occur when normal cells are stimulated into cell division from a quiescent non-dividing state (Baserga *et al.*, 1971; Cooper, 1971), and it is probable that the cell surface plays an important role in this process (Nicolson, 1976; Rozengurt, 1976). However, little attention has been paid to the possible role of the cell surface in exit from the cell cycle, despite the fact that this latter function is possibly the most important property of tumour cells (Stoker, 1972). The purpose of the present study was therefore to compare the surface proteins of normal dividing cells with those of their non-dividing 'switched-off' progeny. The thymus has been used for the present study, since the thymocyte population can be broadly divided into two categories. Larger cells constitute approx. 15–20% of the total (at age 1–2 weeks) and are actively dividing, whereas the progeny of these cells constitute the majority population of small non-dividing cells (Metcalfe & Wiadrowski, 1966; Droege & Zucker, 1975;

Shortman & Jackson, 1974). More recently it has been possible to separate the large- and small-cell populations to high purity (Salisbury *et al.*, 1979), and the two cell types have been used for studies on their surface proteins.

The present paper describes the results of radio-labelling the cell-surface proteins with [¹²⁵I]iodide, by using 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Iodogen) as the catalyst for the reaction (Fraker & Speck, 1978). This compound can be coated on to reaction vessels from solution in dichloromethane and, being water-insoluble, has the advantage that it is not free to enter any cells that may have become 'leaky' and hence cannot cause iodination of internal components.

Experimental

Fractionation of thymocytes

In each experiment, thymus glands from about 25 Wistar-Furth rats or Balb/c mice (aged 8–14 days) were used. The preparation of a single-cell suspension and the fractionation of the thymocytes with Percoll (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden) was described previously (Salisbury *et al.*, 1979). The small-cell (>95% small cells) and large-cell (approx. 80–85% large cells) fractions obtained were diluted and washed twice with Dulbecco A phosphate-buffered saline (Dulbecco & Vogt, 1954). The viability of all cells was >95% (by Trypan Blue exclusion).

Iodination of thymocyte cell surfaces

Approx. 5×10^7 – 1×10^8 large cells or 2.0×10^8 – 3.0×10^8 small cells were used in the iodination reactions. The method used was adapted from that described by Fraker & Speck (1978). Iodogen

Abbreviation used: SDS, sodium dodecyl sulphate.

(Pierce and Warriner, Chester, Cheshire, U.K.) was dissolved in dichloromethane at 0.25 mg/ml and 500 μ l portions were dispensed into 50 mm-diameter glass Petri dishes. The solvent was allowed to evaporate off, leaving the Iodogen coated on to the dishes. The cells were added directly to the dishes in 1 ml of phosphate-buffered saline, and 400 μ Ci of carrier-free Na¹²⁵I (approx. 15 mCi/ μ g; The Radiochemical Centre, Amersham, Bucks., U.K.) was added to each one. The dishes were rocked gently for 15 min at room temperature, after which the cells were transferred to 10 ml centrifuge tubes and washed several times with phosphate-buffered saline to remove unbound [¹²⁵I]iodide.

Membrane preparations

All procedures were carried out at 4°C. Cell pellets from the iodination reaction were resuspended in 5 ml of BMC buffer (1 mM-NaHCO₃ buffer, pH 8.0, containing 1 mM-MgCl₂ and 1 mM-CaCl₂) and homogenized with a tight-fitting Dounce homogenizer. The homogenate was adjusted to a total volume of 14 ml with 60% (w/w) sucrose in BMC buffer [to give 42% (w/w) sucrose] and layered over a 9 ml cushion of 60% sucrose in BMC buffer. The tubes were centrifuged at 95 000 $g_{av.}$ in a 3 $\times$ 25 ml swing-out rotor for 1 h. The nuclei pelleted at the bottom of the 60% sucrose and the membrane material was seen in a band at the 42%/60%-sucrose interface. To avoid losses, the whole of the 42%-sucrose layer and the interface was removed and diluted 5-fold with BMC buffer. The membrane material was then recovered by centrifugation at 118 000 $g_{av.}$ for 1 h in a 8 $\times$ 50 ml angle rotor. The pellets were either used to prepare samples for SDS/polyacrylamide-gel electrophoresis as described below or else resuspended in BMC buffer and used to assay for

ouabain-sensitive (Na⁺ + K⁺)-dependent ATPase as described by Avruch & Wallach (1971).

SDS/polyacrylamide-gel electrophoresis of membrane samples

The membrane pellets were solubilized in 50 mM-Tris/HCl (pH 6.8)/15% (v/v) glycerol/2% (w/v) SDS/0.2 mM-phenylmethanesulphonyl fluoride. Dithiothreitol (0.1 M) and Bromophenol Blue (0.02%) were added and the samples were heated in a boiling-water bath for 3 min, cooled and stored at -20°C. SDS/polyacrylamide-gel electrophoresis in 7.5% (w/v) acrylamide slab gels was carried out as described by Laemmli (1970). The gels were stained with Coomassie Blue, dried, and exposed to Kodak Industrex C X-ray plates.

Determination of ¹²⁵I radioactivity in fractions

Aliquots were taken at different stages throughout the cell rupture and membrane preparation and assayed for trichloroacetic acid-precipitable and total radioactivity. The former was determined by precipitating with cold 5% (w/v) trichloroacetic acid and by filtering and washing through Whatman GF/C discs. The latter was determined by spotting directly on to GF/C discs. In each case the discs were dried and counted for radioactivity in an LKB gamma counter.

Results and discussion

Distribution of ¹²⁵I radiolabel among subcellular fractions

Phase-contrast microscopy showed that all the nuclei from both small- and large-thymocyte homogenates were found in the pellet from the discontinuous sucrose gradient and not in the interface material. Table 1 shows that most of the acid-precipitable radiolabel was associated with the

Table 1. Distribution of ¹²⁵I radiolabel among subcellular fractions of rat thymocytes

Cell population	Fraction	Total radioactivity (as % of ¹²⁵ I radioactivity in reaction mixture)	Macromolecular radioactivity (as % of total radioactivity in fraction)	Macromolecular radioactivity (as % of macromolecular radioactivity in homogenate)
Small cells	1. Homogenate	4.0	16	100
	2. Sucrose gradient:			
	(a) 42% (w/w) sucrose + interface		13	62
	(b) Pellet (nuclei)		42	21
Large cells	3. 118 000 g pellet (membranes)	6.0	48	39
	1. Homogenate		20	100
	2. Sucrose gradient:			
	(a) 42% (w/w) sucrose + interface		12	68
	(b) Pellet (nuclei)		34	19
	3. 118 000 g pellet (membranes)		41	43

Table 2. Ouabain-sensitive Na^+/K^+ -ATPase activity in nuclear and membrane samples from rat thymocytes

Fraction	Na^+/K^+ -ATPase	
	Total activity (μmol of ATP hydrolysed/min)	Specific activity (μmol of ATP hydrolysed/min per mg of protein)
Nuclei	560	0.075
Membrane pellet	1068	0.279

membrane fraction (62–68%), whereas only 19–21% was found in nuclei. These results compare very favourably with other reported data (Crumpton *et al.*, 1976; Juliano & Behar-Bannelier, 1975) for radioiodination of cells in suspension. Despite the apparent absence of unbroken cells and large membrane fragments in the nuclear pellets (as determined by phase-contrast microscopy), the possible presence of plasma membrane in the nuclear fraction was further checked by assay of ouabain-sensitive Na^+/K^+ -ATPase. The results are shown in Table 2; they demonstrate that 34% of the total Na^+/K^+ -ATPase was associated with the nuclear pellet. Hence most of the ^{125}I radioactivity observed in the nuclear fraction was probably due to plasma-membrane contamination rather than to iodination of the nuclear proteins themselves.

Table 1 also shows that 80–84% of the total radioactivity was acid-soluble. Even in the final membrane pellets, the acid-precipitable radioactivity was never higher than 50% of the total. Similar results were reported by Crumpton *et al.* (1976) for lactoperoxidase-catalysed ^{125}I labelling of thymoma cells. They showed that no more than 20% of the total radioactivity could be attributed to labelled lipids and concluded that much of the acid-soluble radioactivity was due to free [^{125}I]iodide. Its presence in the final membrane pellet may be due to either adsorption to the membrane itself or to internalization into membrane vesicles during homogenization.

SDS/polyacrylamide-gel electrophoresis of membrane proteins and autoradiography of the gels

Fig. 1 shows typical results of SDS/polyacrylamide-gel electrophoresis of mouse (a) and rat (b) thymocyte membranes. The experiment was performed several times for each animal and the same results were always obtained. The Coomassie Blue staining (tracks b and c) shows about 35–40 bands, of which the majority are identical for mouse and rat. In both cases a difference occurs between the large and small cells with respect to the relative amounts of three proteins at approx. 45 000–55 000 mol.wt. (marked * in Fig. 1). The autoradiography, however (tracks d and e), shows distinct differences

Fig. 1. SDS/polyacrylamide-gel electrophoresis of membrane samples of mouse (a) and rat (b) thymocytes. Track a shows the position of the marker proteins: β -galactosidase (β -Gal), 130 000 mol.wt.; bovine serum albumin (BSA), 68 000 mol.wt.; creatine kinase (CK), 40 000 mol.wt. Tracks b and c show Coomassie Blue staining of small(b)- and large(c)-cell preparations; tracks d and e show the results of autoradiography of ^{125}I -labelled proteins of small (d) and large (e) thymocytes.

between the mouse and the rat. Broadly, the labelling patterns agree with the results of Standring *et al.*, (1978) for ^{125}I labelling of unfractionated mouse and rat thymocytes, although those authors found a band at approx. 80 000 mol.wt. for both animals that is absent in our autoradiographs. However, they used the lactoperoxidase/glucose oxidase-catalysed method of iodination (Walsh & Crumpton, 1977) and used whole cells for the preparation of gel samples. When we used this method we also

obtained a heavily labelled band at 88000 mol.wt. for all cell types, which we concluded was due to lactoperoxidase and/or glucose oxidase, both of which have apparent mol.wts. of approx. 88000 on SDS/polyacrylamide-gel electrophoresis. This phenomenon was also reported by Monneron & d'Alayer (1978) for ^{125}I -labelled calf thymocytes, and they suggested that it was due to adsorption of lactoperoxidase to the cell surface.

In the mouse cells, five prominent bands were ^{125}I -labelled (Fig. 1a, tracks d and e), but there was no major difference between the large and small cells. Positive identification of the bands cannot be made without using immunoprecipitation techniques, but, by comparison with the results of Williams *et al.* (1978) and Standring *et al.* (1978), band M1 (155000 mol.wt.) probably corresponds to the leucocyte common antigen, and band M3 (100000 mol.wt.) may represent the W3/13 antigen. Band M8 (26500 mol.wt.) is clearly the Thy-1 antigen, and this protein consistently labelled more heavily on the small cells than on the large cells, so that, in the former case, the minor component at 32000 mol.wt. (M7) is obscured.

The rat cells showed a somewhat different labelling pattern (Fig. 1b, tracks d and e) with at least eight major bands, including the leucocyte common antigen (R1), a complex of proteins (R2 and R3) that may contain the W3/13 antigen, and a relatively faint band at 25500 mol.wt. (R8), which may be weakly labelled Thy-1 antigen, although it has been reported (Standring *et al.*, 1978) that rat Thy-1 antigen has only two tyrosine residues that are not readily available for ^{125}I labelling. Component R7 (32000 mol.wt.), which may be a similar protein to M7, is more strongly labelled in the large cells than in the small cells. The major difference between the two animals, however, is that, in the rat, a group of hitherto-unidentified proteins at 40000–60000 mol.wt. (R4–6) were strongly labelled and show distinct differences between the large and small cells. Interestingly, a 51000-mol.wt. component (R5) is heavily labelled in the small cells but faintly labelled or absent from the large cells. This protein runs in a corresponding position to one of the Coomassie Blue-staining bands mentioned above (labelled * in Fig. 1) of which there is more present in the large cells than in the small cells (i.e. the converse of the ^{125}I labelling). Whereas the difference in Coomassie Blue staining is present in both animals the ^{125}I -labelled bands are only found in the rat cells. It is possible that the mouse proteins do not have exposed tyrosine residues available for iodination (either due to a carbohydrate-rich 'coat' or to lack of tyrosine residues themselves, in exposed areas of the protein molecules). It is unlikely that the lack of labelling in the mouse is due to the utilization of all the available [^{125}I]iodide by

other abundant surface molecules (e.g. Thy-1 antigen), since Table 1 shows that the total ^{125}I radioactivity incorporated in the homogenate was only 4–6% of the total radioactivity (400 μCi) present in the iodination reaction.

Little other evidence exists at present for differences in surface-labelled proteins between dividing and non-dividing cells, although Gahmberg *et al.*, (1976) found that a polypeptide of approx. 57000 mol.wt. was weakly labelled in mouse T- and B-lymphocytes by 'non-specific' reduction with NaB^3H_4 (i.e. without any prior oxidation of the cells). This band was much more heavily labelled in mitogenically stimulated T- or B-blasts. They suggested that it might represent an enzyme such as ornithine decarboxylase, which is known to be strongly activated in growing cells (Morris & Filligame, 1974) and would be reducible by virtue of Schiff-base formation.

The major histocompatibility antigens of the mouse (H-2) and rat (Ag-B) have been shown to run on SDS/polyacrylamide-gel electrophoresis with apparent mol.wts. of 30000–50000 (Gill *et al.*, 1978), and there is some evidence that the expression of histocompatibility antigens may vary at different stages in the cell cycle (Pasternak *et al.*, 1971; Pellegrino *et al.*, 1972). However, it has been widely demonstrated that the majority of both large and small thymocytes express very little histocompatibility antigen at their cell surface (Cantor & Weissman, 1976; Shortman & Jackson, 1974). Further investigation is therefore necessary to characterize the 40000–60000-mol.wt. proteins and determine whether they are involved in the control of exit from the cell cycle.

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