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Reactivity of fluorodinitrobenzene with intact human leucocytes: Examination of sites of action and the molecular entities involved in interaction

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Abstract. The epsilon amino groups of lysine and to a lesser extent amino groups on phosphotidyl ethanolamine and phosphotidyl serine situated on cell membranes of normal human leucocytes have been identified as the sites of dinitrophenylation. *In situ* conversion of 10,000 hydrophylic amino sites into hydrophobic sites on cell surfaces causes conformational changes in cell membranes with exposures of leukaemic cell-specific neo-antigens on tagged cell surfaces.

Keywords. Dinitrophenylated leucocytes; leukaemia-associated antigenicity; hydrophylic-hydrophobic interconversions; dinitrophenylated-epsilon lysine; dinitrophenylated phosphatidyl ethanolamine; dinitrophenylated-phosphatidyl serine.

Introduction

Earlier studies from this laboratory (Sahasrabudhe 1968, 1980; Sahasrabudhe *et al.,* 1971a; Joshi *et al.,* 1981) have shown that normal human leucocytes when complexed with l-fluoro-2,4, dinitrobenzene (FDNB) in proportions of 10^4 molecules of FDNB per cell, acquire *de novo* properties such as increased electronegativity on cell surfaces (Prema *et al.,* 1977), mobility of cell surface macromolecules (Sahasrabudhe, 1980), exposures of receptor sites for Concanavalin A (Con A) and wheat germ agglutinins (WGA) on cell surfaces (Madyastha *et al.,* 1975; Barth *et al.,* 1976), very similar to those exhibited by leukaemic cells. These tagged cells when inoculated into rabbits, horses and humans, elicited humoral and cell-mediated immune responses specific against human leukaemic cells (Sahasrabudhe *et al.,* 1971a, b, 1974; Sahasrabudhe, 1973). This induction of antigenicity in chemically modified normal human cells,

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Abbreviations used: FDNB, l-fluoro-2,4,dinitrobenzene; DNP, dinitrophenylated; [^{14}C]FDNB, uniformly [^{14}C]-labelled FDNB; Con A, Concanavalin A; WGA, wheat germ agglutinins.

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exclusively specific against leukaemic cells, has hitherto been unknown in the past. That the immunological responses were not caused by hapten dinitrophenylated moieties (Karande and Sahasrabudhe, 1978) or by the possible dissimilarities in the HLA profiles of the innoculated human cells has amply been proved and demonstrated (Sahasrabudhe *et al.,* 1972a, b). Since dinitrophenylation of 10,000 sites on cell surface involves *in situ* conversion of 10,000 hydrophylic sites into hydrophobic sites, a conformational change dictated by thermodynamic considerations, takes place pushing the newly formed hydrophobic sites (10,000 of them) into deeper hydrophobic microenvironments of the cell membranes, out of contact from the surrounding aqueous medium. This upheaval causes exposure of new macromolecular entities (which were hidden in the cell membrane in cryptic forms) on cell surfaces. It is these newly exposed macromolecular entities which mimic the leukaemic cell surface characteristics (Sahasrabudhe, 1980).

The proportions of FDNB molecules per cell appears to play a critical role in the exposure of leukaemic cell mimetic characteristics in tagged cell surfaces. Optimum level of tagging for eliciting leukaemia-cell-mimetic properties was shown to be 10,000 FDNB molecules per cell. With a few thousand less molecules or a few thousand more molecules of FDNB per cell the leukaemia specific immunological response is either not elicited or partially elicited (Prema *et al.,* 1978).

In order to understand the mechanism involved and why FDNB molecules in the range of 10,000 molecules per cell are required for transforming normal human cells into leukaemic-cell-mimetic entities, it is necessary to known the sites of action of FDNB on cell membranes and what happens to those macromolecular moieties that get complexed with FDNB in the post *in situ* hydrophilic-hydrophobic interconversion phases. This has been attempted in this communication using [^{14}C]-labelled FDNB.

Materials and methods

Separation of human leucocytes and tagging with [^{14}C]-labelled 1-fluoro-2, 4,dinitrobenzene.

Leucocytes from 'O' group normal healthy human donors were obtained as described earlier (Sahasrabudhe *et al.,* 1972a). Radioactive 1-fluoro 2, 4,dinitrobenzene uniformly labelled with [^{14}C] (specific activity 17 mCi/m mols) hereafter referred as [^{14}C]-FDNB, was obtained from Radiochemical Centre, Amersham, England. It was dissolved and diluted with normal saline to give two solutions having concentrations of 10^{14} molecules of labelled FDNB per ml and 10^{16} molecules of labelled FDNB per ml. One ml of each of the above solutions was added to two 1 ml aliquots of leucocytes cell suspensions containing 10^{10} leucocytes per ml. These mixtures were gently shaken for 10 min at room temperature and the tagged cells separated by centrifugation at 500 *g* for 10 min. Supernatant solution was discarded and the FDNB-tagged cell pellets were washed three times with normal saline (each time with 50 ml) to remove unreacted FDNB along with any FDNB that might have combined with loosely bound proteins on cell surfaces (Gharpure, 1977). The washed cell pellets were

resuspended in sucrose, Tris, magnesium chloride buffer (pH 7.2) containing sucrose 0.25 M, Tris 0.01 M and magnesium chloride 0.005 M (Warren and Glick, 1967) and homogenized. The homogenate was centrifuged at 500 *g* for 10 min for the separation of nuclear material. The nuclei-free supernatant was centrifuged at 7500 *g* for 10 min in a Sorval RC-50 centrifuge to separate mitochondrial preparations. The supernatant after removal of mitochondrial pellet was centrifuged for 2 h at 105,000 *g* in a Spinco 65 B ultracentrifuge. The pellet obtained was further fractionated by a second ultracentrifugation in a sucrose gradient as described by Warren and Glick (1967) to obtain the membrane fraction.

Radioactivity in each of the above mentioned fractions was determined by counting aliquots in Beckman LS 100 Liquid Scintillation spectrometer.

Analysis of membrane proteins

Labelled dinitrophenylated (DNP-) membrane proteins were precipitated from the membrane fraction by the addition of 10% cold trichloroacetic acid. Cold (*i.e.* non-radioactive) DNP-membrane proteins were isolated from leucocytes tagged with non-radioactive FDNB using identical procedure with that used in the isolation of labelled DNP-membrane proteins and added as carriers to the latter. The mixtures were hydrolysed with 6 N HCl as described by Lederer and Lederer (1955) . Fifty µg aliquots of membrane protein hydrolysates dissolved in 0.1 ml of methyl ethyl ketone-saturated water were chromatographed on Kiselghur column (Perrone, 1951). The column was gradually eluted with decreasing concentrations of methyl ethyl ketone in chloroform (75%, 45%, 30% and 0%), methanol and water. Absorbance at 360 nm of all the eluate fractions was measured in a Beckman Spectrometer model 26. Fractions showing absorbance at 360 nm greater than 0.05 were concentrated and their radioactivity determined. Individual DNP-amino acids, obtained either from protein hydrolysates or those eluted from various fractions collected after column chromatography, were further identified by chromatography on Whatman No. 3 filter paper with authentic samples of DNP-amino acids in at least two solvent systems (Smith, .1960). DNP-amino acids are easily recognised on filter paper chromatograms by their yellow colour. Epsilon DNP-lysine and DNP-arginine have Rf values in the same range. To distinguish one from the other, ninhydrin spraying was used. Lysine gives brown colour with ninhydrin.

Analysis of membrane lipids

Aliquots of dinitrophenylated cells were centrifuged at 500 *g* for 10 min and the pellet thus obtained was resuspended in normal saline. To this, 20 volumes of chloroform: methanol (1:1) mixture was added and the mixture shaken vigorously to extract the lipids. The chloroform-methanol layer containing the lipids was seperated and the solvent evaporated to dryness. The lipid residue was taken in minimum quantity of methanol and chromatographed on silica gel using chloroform:methanol:water (150:50:7 v/v) solvent system. DNP-phospho-lipids were identified by yellow colour while the unreacted amino-lipids were identified by staining with ninhydrin. The yellow spots containing DNP-phospholipids were scraped of and dissolved in methanol. These were scanned at

345 nm for absorption which is specific for DNP-phosphatidyl serine and DNP-phosphatidyl ethanolamine (Harris *et al.,* 1954). These were further identified by their Rf values and UV absorption spectra. To determine the radioactivity in individual spots, the material was taken in 10 ml Bray's scintillation fluid (naphthalene 18g, 2,5,diphenyloxazole (PPO) 1.2 g 2,2'-*p*-Phenylene-bis-(5-phenyloxazole) (POPOP) 0.05 g dissolved in 1-4 dioxan 264 ml, methanol 30 ml and ethylene glycol 6 ml) and radioactivity counted.

Results and-discussion

When ratio of molecules of FDNB per cell was 10,000, only 46% of the added radioactivity was incorporated in cellular components. When the proportion of FDNB molecules added was increased to 10^6 per cell, the fraction incorporated into cell components decreased to 31 %. The remaining 54% and 69% radioactivity respectively, were lost either as unreacted FDNB or in the form of complex with loosely bound surface proteins which leached out during washing (Gharpure, 1977). Table 1 shows that major portions of radioactivity was present in the membrane fraction when tagged cells bearing 10^4 molecules of FDNB per cell were prepared. Mitochondrial, nuclear and cytoplasmic fractions also incorporated low amounts of radioactivity and could be an artifact of the method.

Table 1. Distribution of radioactivity in the subcellular fractions of leucocytes tagged with [^{14}C]-FDNB.

	10^4 molecules of [^{14}C]-FDNB per cell		10^6 molecules of [^{14}C]-FDNB per cell	
	cpm		cpm	
Whole cells	8400	(46%)[a]	495,562	(31%)[a]
Membrane fraction	5400		7,200	(1.4%)[b]
Mitochondrial	1200	(14%)[b]	211,700	(43%)[b]
Nuclear	1100	(13%)[b]	154,200	(31%)[b]
Cytoplasmic	700	(8%)[b]	122,462	(25%)

Total radioactivity used in the case of 10^4 and 10^6 molecules of FDNB were 18,000 and 1,586,475 cpm respectively. Out of the total radioactivity, 54 and 69% was lost as unreacted FDNB or that complexed with loosely bound proteins in the washings.

[a] % of total radioactivity used.

[b] % of radioactivity incorporated into whole cells.

In the preparation of tagged cells, a calculated amount of FDNB was added to a fixed number of cells expecting that all the cells would have the same number of FDNB molecules per cell. In actual practice, it is likely that while majority of cells would correspond to the desired proportion of FDNB molecules per cell, some cells may have acquired more or some less number of FDNB molecules per cell. The cell membranes were postulated to contain 10^5 amino groups on cell surfaces

(Mehrishi, 1970,. If all the cells had been tagged at 10^4 FDNB molecules per cell, then the chances of FDNB reacting with mitochondrial or nuclear or to cytoplasmic fractions would be nil. The observation that these fractions contain some radioactivity even when the proportion of 10^4 molecules of FDNB per cell was used, suggests that some cells may have reacted with more than the predicted number of FDNB molecules per cell thus enabling FDNB molecules to enter the cytoplasm etc. When the proportion of FDNB molecules added per cell was increased one hundred-fold *i.e.* to 10^6 FDNB molecules per cell, only marginal increase in radioactivity in membrane fraction was seen, whereas the radioactivity in mitochondrial, nuclear and cytoplasmic fractions increased by 150 to 200 fold (table 1).

Distribution of radioactivity in various DNP-amino acids and DNP-phospholipids is shown in figures 1 and 2 and table 2. Figure 1 shows the elution pattern of DNP-amino acids (absorbance at 360 nm) in the hydrolysates of proteins obtained from membrane fractions. Figure 1 shows one major and two

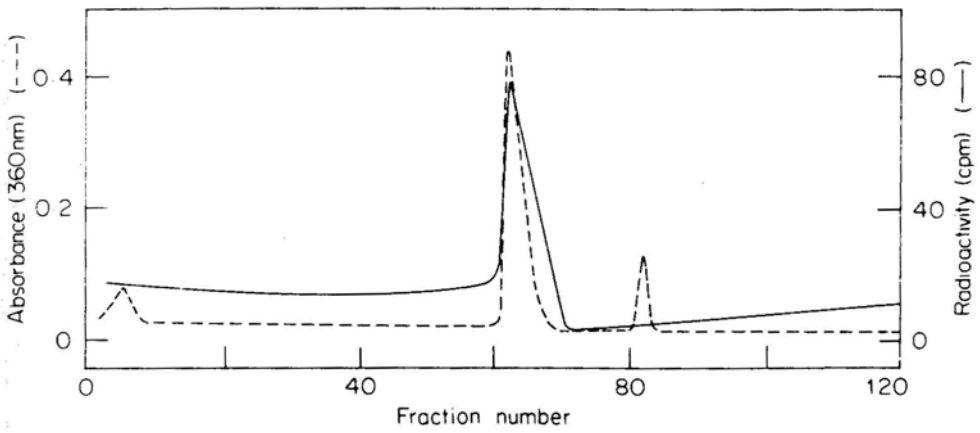

Figure 1. Chromatography of the hydrolysate of FDNB-treated membrane proteins. Membrane protein hydrolysates from dinitrophenylated human leucocytes were chromatographed on Kiesulghur column and absorption at 360 nm (for DNP-amino acids, dotted line in the figure) and radioactivity (solid line in the figure) determined in the eluted fractions.

minor 360 nm absorption peaks corresponding to DNP-amino acids. The major peak which also showed radioactivity was shown to be due to dinitrophenylated epsilon lysine. This fact was confirmed by running the samples along with authentic DNP-amino acid samples on paper chromatography and their reaction with ninhydrin (figure 2). The remaining two minor peaks exhibiting absorption at 360 nm and showing insignificant radioactivity, could not be identified as they were present in trace amounts.

DNP-phospholipids obtained from the membrane fractions were separated by thin layer chromatography and the amounts of DNP-phosphatidyl serine and DNP-phosphatidyl serine and DNP-phosphatidyl ethanolamine were estimated by their absorption at 345 nm. The amounts of the two DNP-phospholipids and their respective radioactivities are given in table 2.

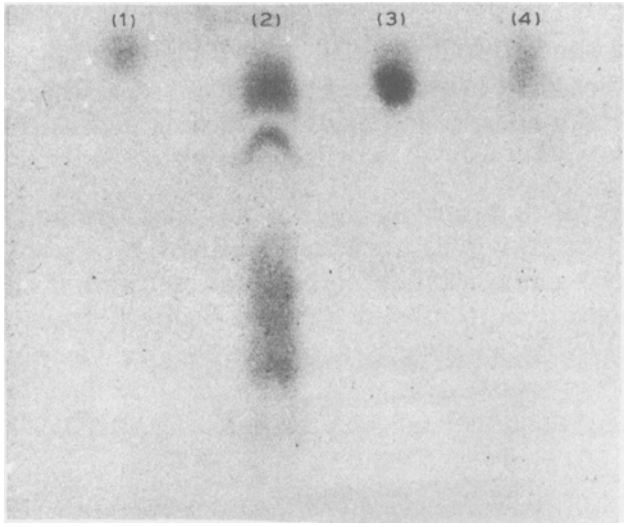

Figure 2. Paper chromatography of membrane protein hydrolysates from dinitrophenylated human leucocytes. 1. "Major peak" eluate from kiesulghur column chromatography (see figure 1), 2. Total membrane protein hydrolysate, 3. ε-Amino dinitrophenylated lysine, 4. Dinitrophenylated arginine.

Table 2. Dinitrophenylation of membrane phospholipids in leucocytes tagged with [^{14}C]-FDNB.

	10^4 molecules of [^{14}C]-FDNB per cell		10^6 molecules of [^{14}C]-FDNB per cell	
	Absorbance (345 nm)	Radioactivity (CPM)	Absorbance (345 nm)	Radioactivity (CPM)
DNP-Phosphatidyl ethanolamine	0.357	180±12	0.469	217±29
DNP-Phosphatidyl serine	0.174	106±10	0.277	96±9

These findings indicate that with 10,000 molecules of FDNB per cell *(i.e.* the proportion which exposes *de novo* leukaemic cell mimetic properties on cell surfaces) the amount is just sufficient to dinitrophenylate only the membrane constituents. With larger amounts, more FDNB is available for entry into the cell for dinitrophenylation of mitochondria, nuclei and cytoplasmic constituents. But this adversely affects the capacity of tagged cells to exhibit its leukaemia antigenicity (Prema *et al.,* 1978). Apparently epsilon amino group of lysine and to a lesser extent phosphatidyl ethanolamine and phosphatidyl serine are the only constituents in the membrane which were dinitrophenylated. Dinitrophenylation of phospholipids, as has been stated earlier, is considered to be an artifact arising from cells which have reacted when a higher amount of FDNB molecules per cell than the desired 10,000 molecules was used. The exact mechanism of exposure of leukaemia specific neo-antigens on tagged cells and the role dinitrophenylation of epsilon lysine plays in these transformations is not clear. That the DNP-hapten has no role in creation of leukaemia antigenicities has been shown by the fact that tagged cells do not respond to specific anti-DNP antibodies (Karande and Sahasrabudhe, 1978). By dinitrophenylation, we are converting *in situ* 10,000 hydrophylic groups on cell surfaces into an equal number of hydrophobic groups (Iyer *et al.,* 1979, Haseley and Biltonen, 1975). The presence of 10,000 hydrophobic groups on cell surface in contact with aqueous environment would require expenditure of considerable energy, without which there would be a natural tendency for the newly formed hydrophobic groups to sink deeper into the inner hydrophobic microenvironment (Singer, 1971). This *in situ* conversion of a large number of hydrophilic sites into hydrophobic site would cause conformational changes and possibly convulsions in cell surface topography causing exposure of neoantigens on tagged cell surfaces which mimic leukaemia-associated antigenicity.

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