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FLUORESCENT LABELLING OF PROTEINS OF LYMPHOCYTE PLASMA MEMBRANES

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Fluorescamine has been used for labelling proteins present on the surface of normal human peripheral blood lymphocytes. Under the conditions of study, 12 labelled proteins could be detected by SDS gel electrophoresis. This method may be of value in biochemical studies of lymphocyte membranes.

INTRODUCTION

In order to understand the molecular basis of lymphocyte responses, it is necessary to characterise the protein structures in the plasma membrane. A major difficulty in isolating and characterising proteins from the plasma membrane of lymphocytes is obtaining material sufficiently homogeneous with respect to lymphocyte surface properties. One approach has been the development of established cell lines of lymphoid cells derived either from normal subjects or from lymphoproliferative disorders, but such cells may display abnormalities such as chromosome variations and new membrane properties (Steel and Hardy, 1970; Dick et al., 1972). An alternative method has been the development and use of 'non-permeant' label reagents. The detection of label is dependent on the type of reagent used. Fluorescent and radioactive groups are measured by fluorimetry and scintillation counting or autoradiography, respectively. The most commonly used method of membrane labelling is lactoperoxidase iodination of surface proteins.

Fluorescamine is a reagent which interacts with the primary amino groups of proteins to yield highly fluorescent derivatives (Udenfriend et al., 1972). The reaction proceeds at room temperature with a half-time of a fraction of a second. Excess reagent is concomitantly destroyed with a half-time of several seconds (Udenfriend et al., 1972). Fluorescamine and its hydrolysis products are non-fluorescent.

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Fluorescamine has already been used for the fluorimetric assay of proteins in amounts in the nanogram range (Bohlem et al., 1973) and a very and precise amino acid analyser method making use of fluorescamine in place of ninhydrin has also been described (Stein et al., 1973). Recently fluorescamine has been used for labelling the surface proteins of erythrocytes and fibroblasts (Nakaya et al., 1975; Hawkes et al., 1976).

In this paper we describe the use of fluorescamine for covalent labelling of proteins present on the surface of normal lymphocytes. We also give preliminary results for detection of these fluorescent proteins by SDS gel electrophoresis.

MATERIALS AND METHODS

The sources of chemicals were: acrylamide, 2-mercaptoethanol, Sigma; ammonium persulphate, N,N,N′-tetramethylenediamine, bisacrylamide, Serva; sodium dodecyl sulphate, Matheson, Coleman and Bell Technical; fluorescamine, Hoffman-La Roche; Lymphoprep, Nyegaard; Coomassie brilliant blue R 250, BDH; Lipid Crimson, Searle Diagnostic.

Lymphocyte separation

Defibrinated human peripheral blood was mixed with an equal volume of sodium chloride (0.9%) and then layered on Lymphoprep and centrifuged at $400 \times g$ for 35 min. The interface cells were carefully washed 3 times with phosphate-buffered saline (PBS) and counted. The cells consisted of lymphocytes, some monocytes and less than 1% polymorphs.

Fluorescence labelling procedure

Ten million lymphocytes in 0.5 ml PBS were incubated with 10 μl of fluorescamine (2 mg/ml) in dimethylsulphoxide with continuous shaking. Immediately PBS with foetal calf serum was added and the cells were washed and then solubilised in 1 ml 0.0625 M Tris-HCl pH 6.8 containing 2% sodium dodecyl sulphate (SDS) and 2% 2-mercaptoethanol in a boiling water bath for 5 min. Glycerol (1/10 vol) was added to each sample solution.

SDS-acrylamide gel electrophoresis

Electrophoresis was performed according to the method of Laemmli (1970). Samples of labelled proteins (10—100 μl) equivalent to 10^5—10^6 cells were applied to each gel column (12 cm × 0.5 cm) consisting of 8% (w/v) polyacrylamide. Low concentrations of ammonium persulphate 0.08% (w/v) and N,N,N′-tetramethylenediamine 0.08% (v/v) were used to catalyse the reaction.

Electrophoresis was performed at room temperature with a constant current of 1.5 mA per tube until the tracking dye (bromophenol blue) moved to approximately 0.5 cm from the end of the gel. One of the gels was stained with 5 ml of 1% Coomassie brilliant blue R 250 solution made up in 45 ml

methanol, 15 ml acetic acid and 40 ml dist. H_2O. The gels were destained with a solution containing 30 ml acetic acid, 40 ml methanol and 430 ml dist. H_2O, until the contrast between band and background became sharp. Lipid Crimson staining for lipoproteins was done according to Smith (1976).

Fluorometric method

After the run the gels were frozen and sliced in 40 fractions with a slicer. The proteins were eluted from the gel slices by adding 1 ml of 0.1% SDS and shaking for 12 h at 37° C (Weber and Osborne, 1969; Vandekerckhove and Van Mouragu, 1974). Then 3.5 ml dist. H_2O was added and fluorescence was measured with a Perkin-Elmer fluorescence spectrophotometer with excitation at 390 nm and emission at 475 nm.

RESULTS AND DISCUSSION

As can be seen in Fig. 1, 12 fluorescent bands were resolved after fluorescamine labelling of living lymphocytes. All experiments have been repeated up to 5 times with reproducible results. The consistency of results was confirmed by running in SDS two fluorescamine labelled samples of lymphocytes from the same donor labelled under similar conditions. As is shown in Fig. 2, nearly identical patterns were obtained. Fig. 3 shows typical ring staining of a lymphocyte labelled with fluorescamine, similar to the staining pattern seen for membrane Ig.

In solubilised fractions of labelled cells kept in the dark at −20° C for up to 3 weeks fluorescence was stable over this period.

On staining the gels with Lipid Crimson only one band was detected, very close to the end of the gel. This may indicate that band No. 12 represents

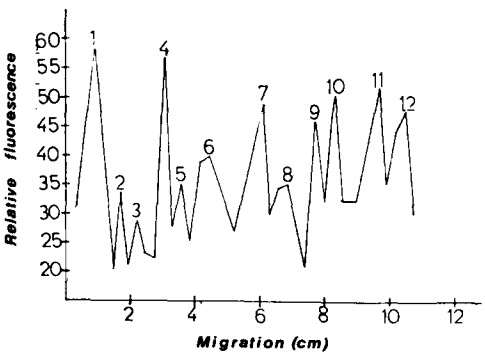

Fig. 1. SDS acrylamide gel electrophoresis pattern of fluorescamine labelled proteins from normal human lymphocytes.

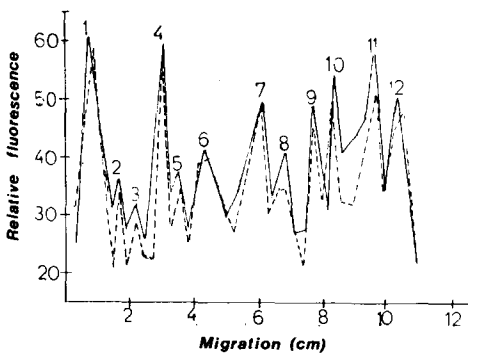

Fig. 2. Comparison of the patterns of two lymphocyte samples labelled with fluorescamine under similar conditions.

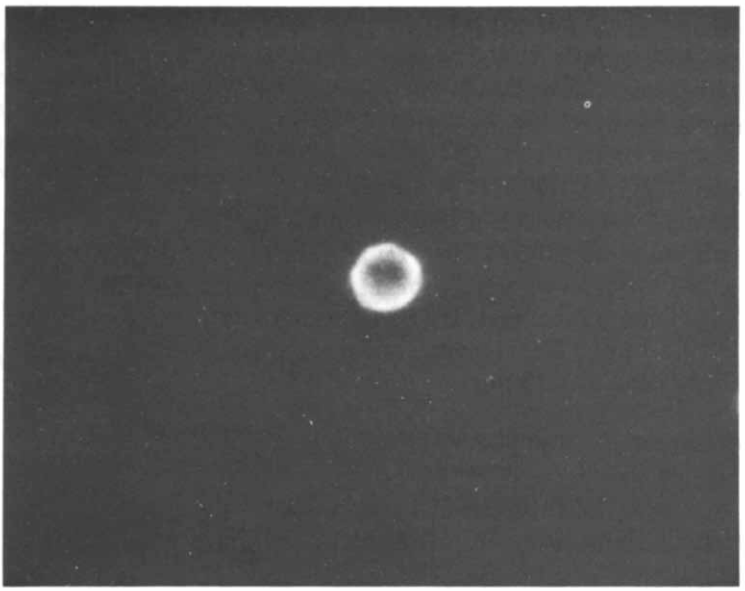

Fig. 3. Surface staining of a lymphocyte with fluorescamine.

fluorescamine labelled phospholipids present in lymphocyte membranes. A similar fluorescent band produced by the reaction between dansyl chloride and lipids was also reported by Schmidt-Ullrich et al. (1973).

The object of this study was to label with fluorescamine proteins associated with the surface of living lymphocytes. Under the conditions described 12 bands were detected. However, when gels were cut into 80 slices 16 bands could be traced (data not shown here).

Dead cells are labelled interiorly, showing a diffuse staining pattern under the immunofluorescence microscope. Thus good viability is necessary in this procedure.

Because of the complexity of cell structure of lymphoid cells compared with erythrocytes, the question whether labelling is taking place at the cell surface needs to be considered. Fluorescamine labelling is very rapid and the typical ring staining of cells seen under the immunofluorescence microscope is analogous to that of surface Ig on B lymphocytes. Our observation that the same number of fluorescent bands were labelled in separate experiments probably indicates that the same population of cell membrane proteins were being labelled in a reproducible manner. If cytoplasmic proteins had been labelled as well, this would have produced extra bands of fluorescence. Hawkes et al. (1976) and Nakaya et al. (1975) using fibroblasts and erythrocytes respectively found that intracellular proteins were not labelled.

The selectivity of lactoperoxidase iodination for labelling proteins only on the outer surface of plasma membranes is controversial. In contrast with the results of Marchalonis et al. (1971), Schmidt-Ullrich et al. (1974) found that

^{125}I activity was not exclusively associated with the plasma membrane of thymocytes but also occurred in other subcellular fractions. Podulso et al. (1972) have reported significant labelling of lipids by lactoperoxidase treatment, with approximately 40% of the radioactivity associated with the cytoplasmic contents of cells.

Another disadvantage of lactoperoxidase iodination compared to that of fluorescamine is that the labelling is restricted to proteins having exposed tyrosine, or possibly histidine, residues.

Using fluorescamine, labelling occurs at physiological tonicity and pH. Furthermore, this method detects proteins present on the surface of lymphocytes at very low concentrations. The procedure also obviates the necessity of isolating membrane with its attendant difficulties and ambiguities.

Fluorescamine labelling has been applied to the study of erythrocyte membrane surface proteins by using cycloheptaamylose-fluorescamine complex (Nakaya et al., 1975).

With fibroblasts labelled with fluorescamine Hawkes et al. (1976) had difficulty in precisely locating fluorescent bands since a hand-held UV lamp was used; under these conditions only two major areas of fluorescence could be seen making resolution of surface proteins impossible.

We believe that the fluorescamine labelling procedure may be helpful in defining differences in surface proteins between normal lymphocytes and those present in lymphoproliferative disorders.

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