The use of horseradish peroxidase–lectin conjugates to monitor the purity of plasma-membrane preparations obtained from isolated cells

J. Paul BANGA, Philip PENFOLD and Ivan M. ROITT

Department of Immunology, Middlesex Hospital Medical School, Arthur Stanley House, 40–50 Tottenham Street, London W1P 9PG, U.K.

(Received 19 March 1981/Accepted 22 April 1981)

The plasma membranes of viable murine EL4 tumour cells were labelled with horseradish peroxidase-conjugated wheat-germ agglutinin. After disruption of the labelled intact cells, plasma-membrane purification could be monitored by ultrastructural examination of the various fractions for positive reaction product on the membrane vesicles.

The molecular analysis of plasma-membrane fractions cannot be undertaken usefully until one has a means of assessing their purity. In most instances, this has been ascertained from enzymic activity, in particular 5'-nucleotidase, but other criteria such as morphology, antigenic markers, buoyant density and cholesterol/phospholipid ratio have also proved to be useful parameters (Crumpton & Snary, 1974). However, inherent difficulties such as variation in enzymic activity of the same tissue in different species, in localization of the assayed enzyme in different cells and differential buoyant density of plasma membrane from different cell types is likely to be responsible for the widely reported values of membrane purity reported in the literature (Evans, 1979). We now describe a procedure for determining membrane purity of isolated cells that involves the labelling of intact cells with peroxidase-conjugated lectin, and ultrastructural examination of the plasma-membrane preparation for positive reaction product.

Methods

The murine T-cell tumour EL4 was maintained by serial passaging in C57/B1 mice. The horseradish peroxidase–wheat-germ agglutinin conjugate was prepared by the method of Avrameas (1969). Briefly, $4 \times 5 \mu\text{l}$ portions of 1% (w/v) glutaraldehyde solution were added to 2 mg of wheat-germ agglutinin (Pharmacia Fine Chemicals) and 5 mg of horseradish peroxidase (Miles Laboratories) in 400 μl of 0.1 M-sodium phosphate buffer, pH 6.8. After 2 h at room temperature, the sample was extensively dialysed at 4°C against phosphate-buffered saline and the conjugate was stored in the presence of 0.1% (w/v) glycine at 4°C. Before use,

any precipitate that formed was removed by centrifugation in a bench Microfuge.

A suspension of EL4 cells ($\geq 98\%$ viable) in Hanks' balanced salt solution containing 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and 0.02% sodium azide (0.5×10^7 cells/cm³) was labelled on ice with various concentrations of horseradish peroxidase–wheat-germ agglutinin to determine the optimal concentration of conjugate required to label the entire cell surface adequately without causing agglutination. For EL4 cells with horseradish peroxidase–wheat-germ agglutinin, this was found to be 100 μg of horseradish peroxidase–wheat-germ agglutinin/ 0.5×10^7 cells per cm³. Occasionally a small number of cells agglutinated, as judged at the light-microscope level, and these were disaggregated by vortex-mixing.

Usually a total of 10^8 EL4 cells in 20 cm³ of the balanced salt solution containing 0.02% sodium azide were labelled with 2 mg of horseradish peroxidase–wheat-germ agglutinin. After labelling, the cells were left on ice for 30 min and pelleted by centrifugation at 800 g_{av} for 3–4 min. The labelled cells were then resuspended in 2 cm³ of cold balanced salt solution containing 0.02% sodium azide at a concentration of 5×10^7 cells/cm³ and a 50 μl portion removed for electron microscopy. The remaining cells were disrupted with a Stansted model A0612 cell disrupter (Stansted Fluid Power) (Crumpton & Snary, 1974) using disrupting valve 716. The optimal pressure for disruption of EL4 cells was found to be 1200 kPa. After disruption, the plasma membrane was separated by differential and sucrose-density-gradient centrifugation in accordance with the scheme developed by Crumpton & Snary (1974). For electron microscopy, cells labelled with conjugate and membrane pellets were fixed in 2% (w/v) glutaraldehyde and 0.1 M-cacodylate buf-

fer, pH 7.4. The histochemical procedure for the demonstration of peroxidase reactivity was carried out by the method of Graham & Karnovsky (1966). The specimens were then post-fixed in OsO_4 , dehydrated and embedded in Araldite. Sections were examined in an AE1 EM6B electron microscope at an accelerating voltage of 60 kV.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed in a 5–15% (w/v) polyacrylamide gradient that was prepared and electrophoresed in the Laemli buffer system as described by Thorpe *et al.* (1979). The membrane material was prepared for electrophoresis (Thorpe *et al.*, 1979) and a sample of 40–50 μg of protein was loaded.

Results and discussion

Various lectin–horseradish peroxidase conjugates were assessed for their ability to label the entire surface of EL4 cells. Horseradish peroxidase–wheat-germ agglutinin was found to be superior to horseradish peroxidase conjugates of concanavalin A, succinyl-concanavalin A and *Lens culinaris* lectin in its ability to give a strong peroxidase reaction product uniformly distributed over the entire surface of EL4 cells (Plate 1a). This correlates well with our previous observations that fluorescein-labelled wheat-germ agglutinin stains all EL4 cells. After labelling under sub-agglutinating conditions, the cells were disrupted and a purified plasma-membrane preparation was isolated. When examined under the electron microscope, a high proportion of the membrane vesicles showed positive labelling with the horseradish peroxidase reaction product (Plate 1b); the purity of the plasma membrane was determined to be $\geq 90\%$. A control plasma-membrane fraction obtained from cells treated with horseradish peroxidase coupled to bovine serum albumin showed no labelling with the conjugate (Plate 1c). In contrast, the microsomal pellet from the horseradish peroxidase–wheat-germ agglutinin-labelled disrupted cells contained both labelled and a large proportion of unlabelled membrane fragments (Plate 1d).

Our interpretation that the labelled membrane is derived from the cell surface and the unlabelled membrane represents intracellular membrane is based on the following arguments. First, the labelling was performed with highly viable cell populations on ice in the presence of sodium azide and this therefore discounts the possibility that endocytosis of the capped horseradish peroxidase–wheat-germ agglutinin membrane glycoprotein(s) receptor complex might be responsible for labelling intracellular membranes. Furthermore, examination of labelled intact cells revealed very little intracytoplasmic staining (Plate 1a). Secondly, the presence of clearly

labelled and unlabelled membrane vesicles in the microsomal fraction (Plate 1d) argues against the possibility of bound horseradish peroxidase–wheat-germ agglutinin dissociating from the cell surface during disruption and membrane purification and then rebinding to unlabelled intracellular membranes.

When cells are disrupted, a heterogeneous population of membrane fragments is formed, a proportion of which may reseal, leading to a mixed population of right-side-out and inside-out plasma-membrane vesicles. If the latter are impermeable to the enzyme substrate, diaminobenzidine, the cell-surface-bound horseradish peroxidase–wheat-germ agglutinin conjugate would not generate reaction product. However, the yield of sealed, impermeable vesicles from sucrose-density-gradient-centrifugation-purified membranes may well be small, since detailed studies by Johnstone & Crumpton (1980) have shown that sucrose has an inhibitory effect on their formation. Furthermore, direct evidence in the electron microscope of labelled membrane vesicles (Plate 1b) shows that the majority of the small 'sealed' vesicles are labelled, suggesting that they are either right-side-out or permeable if inside-out and are likely to be derived from the cell surface.

A general approach for membrane preparation that has been used extensively depends on stabilization of the plasma membrane with such diverse compounds as Zn^{2+} , fluorescein mercuric acetate and glutaraldehyde before cell disruption (Evans, 1979). Cell lysis leads to a population of large membrane fragments, the majority of which are not vesiculated. Recently, the lectin concanavalin A has also been shown to act as a membrane stabilizer against fragmentation and vesiculation (Lutton *et al.*, 1979) and it might be argued that in our studies, if horseradish peroxidase–wheat-germ agglutinin behaves in a similar fashion, the final purity of the plasma-membrane fraction could be greater than that of the corresponding unlabelled membrane preparation, since vesiculation in the latter may lead to trapping of cytoplasmic material. However, although there is an increased frequency of unsealed vesicles in the labelled compared with the control plasma-membrane preparations (Plate 1b versus Plate 1c), it is unlikely that trapping of intracellular impurities is significant because labelled and unlabelled membrane preparations show virtually identical polypeptide patterns on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1).

It also seems unlikely that these results on the plasma-membrane fraction have been affected significantly by any change in the buoyant density of the plasma membrane resulting from combination with the horseradish peroxidase–wheat-germ agglutinin. Plasma membranes are recovered from the

EXPLANATION OF PLATE 1

Binding of horseradish peroxidase-wheat-germ agglutinin to EL4 cells and various membrane fractions

(a) Intact cell labelled with 100 μ g of horseradish peroxidase-wheat-germ agglutinin/ 0.5×10^7 cells per cm^3 . The surface is uniformly stained with the black peroxidase reaction product. (b) Purified plasma membrane from (a) showing label on nearly all the vesicles. (c) Purified plasma membrane treated with horseradish peroxidase-bovine serum albumin ($100 \mu\text{g}/0.5 \times 10^7$ cells per cm^3), showing lack of staining. (d) Microsomal fraction from (a). A proportion of the vesicles are stained and presumably represent plasma membrane contaminants. Magnification: (a) 5000 $\times$; (b) 30000 $\times$; (c) 30000 $\times$; (d) 30000 $\times$. Abbreviations used: U, unlabelled membrane (horseradish peroxidase-wheat-germ agglutinin-negative); L, labelled membrane (horseradish peroxidase-wheat-germ agglutinin-positive).

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of (a) EL4 plasma membrane from unlabelled cells, (b) plasma membrane from EL4 cells labelled with horseradish peroxidase-wheat-germ agglutinin ($100 \mu\text{g}/0.5 \times 10^7$ cells per cm^3) and (c) horseradish peroxidase-wheat-germ agglutinin conjugate. The molecular weights ($\times 10^{-3}$) are indicated.

upper sucrose layer after centrifugation of the microsomal fraction on a one-step gradient of 25 and 37% (w/w) sucrose; any membrane of lowered buoyant density would still sediment to the interface between the sucrose solutions where the plasma-membrane fraction is normally located, so that any decrease in buoyant density would not change the purity of the final membrane preparation. Any increase in buoyant density should, if anything, decrease the purity by sedimenting the plasma membrane with the microsomal fraction.

In summary, the binding of labelled lectin to the cell surface does not appear to affect the purity of the final membrane preparation, which can therefore be directly assessed by the staining properties of the individual membrane fragments.

The isolation of highly purified plasma membrane and the reasonably uniform label on the plasma membrane fragments has been achieved with a relatively homogeneous tumour cell population but we have also used this method successfully to evaluate the purity of membrane preparations derived from a heterogeneous population of lymphocytes such as are present in pig mesenteric lymph-node cell suspensions (J. P. Banga, P. Penfold & I. M. Roitt, unpublished work). One disadvantage of the technique is that various peroxidase-conjugated lectins have to be screened to satisfy the requirements for uniform labelling of the entire cell surface of the cells; for example, we used conjugated *Lens culinaris* lectin to label pig lymphocytes. In addition, the method is only suitable for isolated cells.

Clearly, lectins are not the only ligands that can be employed and we are at present looking at the possibility of using monoclonal antibody to human β_2 -microglobulin conjugated to horseradish peroxidase as a universal reagent for assessing the purity of membrane preparations from human lymphocytes.

This study was supported by a grant from the Medical Research Council. We thank Dr. M. J. Crumpton for advice on the use of the Cell Disrupter.

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