

A METHOD FOR ANALYSING LYMPHOCYTE SURFACE ANTIGENS

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A method is described for isolating and characterizing external lymphocyte surface proteins. Intact ^{125}I -labelled tonsillar lymphocytes were incubated with antilymphocyte serum, solubilised with NP-40 precipitated with *Staphylococcus aureus* and the labelled proteins analysed on SDS polyacrylamide gels. The molecular weights of the protein labelled in this way were compared with those of surface antigens labelled by the galactose oxidase method. This method may also be used for isolation of surface receptor molecules which lose their stereochemical structure upon solubilisation of the cells.

INTRODUCTION

Although a number of methods exist for the isolation and characterization of lymphocyte surface antigens most suffer from limitations. In most cases a tracer is used owing to the small quantities of surface proteins present (Bretscher, 1971; Lambris et al., 1979). The most reliable techniques currently in use for labelling cell surface antigens are lactoperoxidase iodination and galactose oxidase labelling. However, after iodination of the lymphocytes as much as 40% of the label was found inside the cells (Podulso et al., 1972). Some limitations of the galactose oxidase method have been outlined by Steck and Dawson (1974).

We have used a new method to study the molecular nature of the external proteins of lymphocytes. Radioiodinated lymphocytes were treated alive with antilymphocyte serum, solubilised with Nonidet P-40 and precipitated with formalin-fixed *Staphylococcus aureus* (SA) and the precipitates were analysed by SDS polyacrylamide gel electrophoresis (PAGE). A comparison of the proteins revealed by this method and the galactose oxidase labelling was also made.

MATERIALS AND METHODS

Lymphocytes

Lymphocytes from tonsils were minced with scissors and passed through a stainless steel mesh to obtain a single cell suspension. Cells were then

layered on Lymphoprep (Nyegaard, Oslo) and the interface cells were washed 3 times and counted.

Production of ALS

New Zealand white rabbits selected for the absence of natural antibodies to human lymphocytes were used. Antisera were raised by injecting intravenously 200×10^6 viable tonsil lymphocytes. Similar booster injections were given 2 weeks later. After 10 days the rabbits were bled by cardiac puncture. The sera obtained were heat inactivated at 56°C for 30 min. Cytotoxicity testing of the ALS was according to Mittal et al. (1968).

Radioiodination of lymphocytes

Cells were radioiodinated by the method of Vitetta et al. (1971). Briefly, $1-2 \times 10^8$ cells in 2 ml PBS were mixed with 1 mCi of [^{125}I]Na (Amersham) and 200 μg of lactoperoxidase (Sigma). Four portions (25 μl each) of freshly prepared 0.03% hydrogen peroxide were added at 2.5 min intervals. After 10 min the reaction was stopped by the addition of 10 ml cold PBS. The cells were then washed 4 times with PBS.

Immunoprecipitation of surface antigens

2×10^7 tonsil lymphocytes were incubated with 20 μl ALS at 4°C for 30 min. Excess ALS was removed by washing the cells with PBS 3 times. The lymphocytes were lysed with 1 ml NP-40 (Shell Company) 0.5% (v/v) in phosphate-buffered saline (PBS) containing 2 mM phenylmethylsulphonyl fluoride and 20 mM iodoacetamide at 0°C for 30 min. The cell lysate was centrifuged at $10,000 \times g$ for 10 min and the supernatant incubated with 200 μl of SA (10% v/v) at room temperature for 60 min, or with an excess of anti-rabbit IgG, for 16 h at 4°C . Normal rabbit serum (NRS) was used as control. Precipitates were pelleted by centrifugation at $3000 \times g$ for 10 min.

Galactose oxidase labelling

Cells were labelled with ^3H by a slight modification of the method of Chamberg et al. (1976) using 5 units galactose oxidase (Sigma), 25 units neuraminidase (BDH), and 1 mCi NaB^3H_4 (Amersham) to 2×10^7 tonsil lymphocytes.

SDS-PAGE

Labelled antigens obtained by immunoprecipitation with ALS and NRS or cells labelled by the galactose oxidase method were analysed by electro-

phoresis in SDS polyacrylamide gels according to the method of Laemmli (1970). Washed immunoprecipitates with PBS or labelled cells resuspended in 100 μ l buffer containing 2% SDS and 100 mM iodoacetamide were heated in a boiling water bath for 10 min. In the case of precipitation with SA the precipitates were washed 4 times with PBS, solubilised as above, and centrifuged at 3000 $\times g$ for 10 min. The supernatant was then applied to the polyacrylamide gel for electrophoresis. After electrophoresis the rod gels containing 125 I-labelled antigens were sliced into sections and counted with an Autogamma counter. Slab gels or rod gels containing standard proteins were stained overnight in a solution consisting of 425 ml methanol, 75 ml glacial acetic, 500 ml water and 0.05% Coomassie blue R 250 (BDH). Gels were destained at 37°C in the same solution without Coomassie blue. Gels containing [3 H]antigens were treated with dimethylsulphoxide/2,5-diphenyloxazole according to Bonner and Laskey (1974) and fluorography was carried out using XR-5 Kodak film. Scanning of fluorographic film was performed with a Gilford gel scanner.

The protein standards used and their molecular weights (MW) were: IgG (160,000), transferrin (76,000), BSA (68,000), ovalbumin (43,000), chymotrypsinogen (25,700) and lactalbumin (19,400). The MWs of the labelled antigens were estimated from log MW vs. mobility curves of the standards.

RESULTS

The results of cytotoxicity testing of the ALS on human lymphocytes are shown in Fig. 1 and indicate that the ALS used is directed against all the subpopulations of lymphocytes present in tonsils.

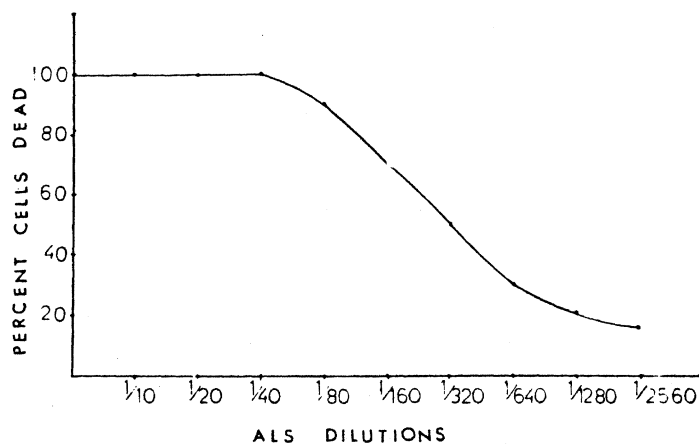


Fig. 1. Cytotoxicity of ALS for tonsillar lymphocytes.

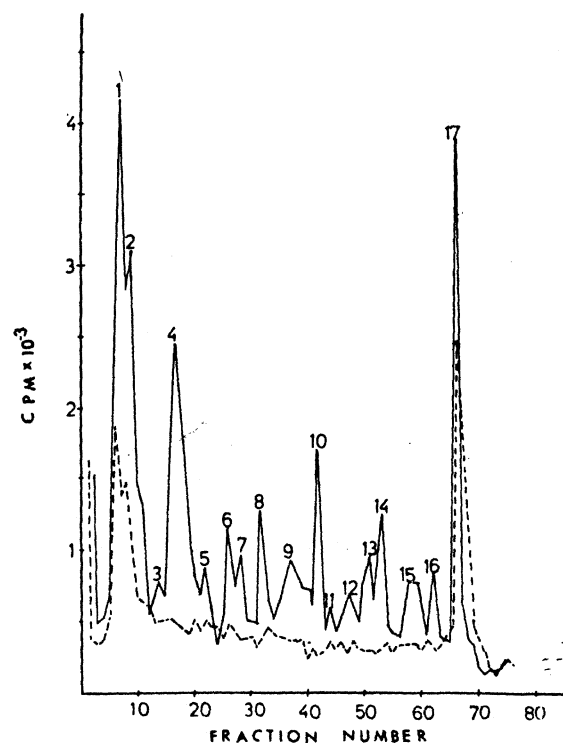


Fig. 2. Migration pattern in 7.5% polyacrylamide gel electrophoresis of surface antigens obtained by specific precipitation with ALS and SA. An aliquot of precipitate solubilised by 2% SDS was layered on polyacrylamide gel. The gel was frozen, sectioned in 1.1 mm slices and counted in an autogamma counter. —, precipitate with ALS and SA; - - - - -, precipitate with NRS and SA.

In Fig. 2 the profile of surface antigens may be seen. Seventeen radioactive bands are detected on ALS treatment of the cells and precipitation with SA, whereas only 3 bands are present when normal rabbit serum is used. It is better to section the rod gels and count the radioactivity of the separate slices rather than prepare autoradiograms of slab gels, since iodinated surface proteins with high radioactivity can overlay weak bands.

In Fig. 3 proteins labelled with the galactose oxidase method are seen. It is evident that approximately the same number of bands are produced by surface iodination and galactose oxidase labelling.

The 3 bands seen with normal rabbit serum (Fig. 2) represent Fc receptors as indicated by preliminary experiments with different cell types (Lambris and Papamichail, in preparation). Bands 16 (Fig. 3) and 17 (Fig. 2) run with the tracing dye bromophenol blue.

Table 1 gives the molecular weights of the iodinated and tritiated surface proteins. It can be seen that with both methods, proteins and/or glycopro-

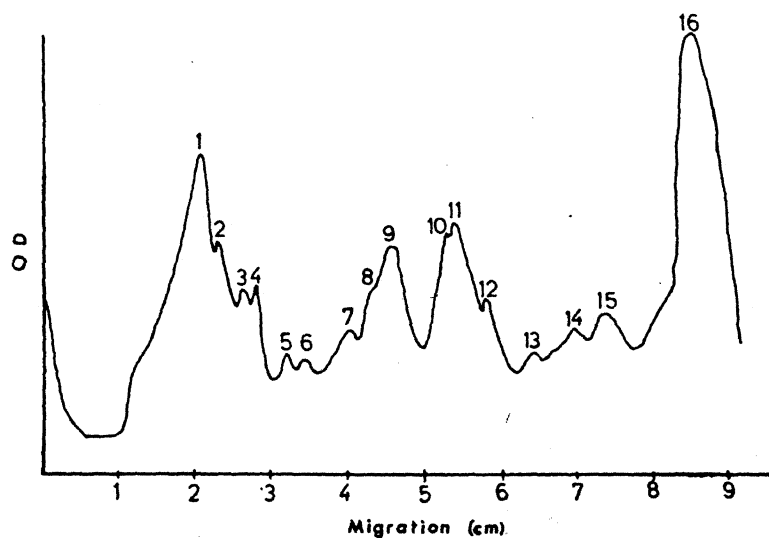


Fig. 3. Migration pattern of labelled external plasma membrane glycoproteins from ^3H -labelled tonsil lymphocytes after electrophoresis in 7.5% polyacrylamide gels, fluorography and scanning of the film by a Gilford gel scanner.

TABLE 1

Molecular weights of ^{125}I immunoprecipitated antigens and ^3H -labelled glycoproteins of human tonsil lymphocytes.

MW $\times 10^3$	^{125}I labelling followed by precipitation with SA	^3H labelling
180	+	—
160	+	+
150	+	+
135	—	+
125	+	+
105	+	+
94	+	+
80	+	+
76	+	+
69	+	+
56	+	+
50	+	+
43	+	+
35	+	+
30	+	+
24	+	+
22	+	—

teins of the same molecular weight range are labelled. Only two bands detected by the radioiodination method are absent when cells were labelled with sodium borohydrate. One glycoprotein (MW 135,000) is not present when cells are labelled with ^{125}I and immunoprecipitation with SA is performed.

DISCUSSION

A method is described for analysis of the profile of lymphocyte plasma membrane associated external antigens. The procedures used are iodination and treatment of the intact lymphocytes with antilymphocyte serum, solubilisation of the cells with non-ionic detergent, precipitation of the ALS reacted proteins with SA and SDS-polyacrylamide gel electrophoresis of the precipitated labelled proteins. The method obviates the labelling of internal proteins which occurs with the lactoperoxidase iodination method (Podulso et al., 1972; Schmidt et al., 1974). Furthermore, the interaction of the antibody with the antigens takes place in the natural environment of these surface molecules, thus avoiding change of tertiary structure of the proteins during solubilisation of the cells.

It should be stressed that careful standardisation and titration of the antilymphocyte serum is essential before the SDS gel electrophoresis is carried out, since overloading of gels due to presence of unlabelled antibody proteins as well as labelled surface proteins may occur. The use of SA for precipitation is rapid, more efficient, and less artifact-prone than precipitation with anti-immunoglobulin reagents. This accords with published work on the characterization of defined surface antigens by immunoprecipitation (Cullen and Schwartz, 1977).

All the antigens immunoprecipitated represent external membrane proteins since the lymphocytes were incubated with ALS alive so that penetration of the membrane did not occur. The method may also be used when, after solubilisation with detergents, surface receptors lose stereochemical properties which are necessary for expression of the biological function of receptors. For example, when cell surface C_3b receptors are solubilised with non-ionic detergent, loss of biological function of the receptors occurs (Dierich and Reisfeld, 1975). However, when cells are first treated live with C_3b and then solubilised, isolation of this receptor is possible (Lambris et al., in preparation).

REFERENCES

- Bonner, M.W. and F.A. Laskey, 1974, *Eur. J. Biochem.* 46, 83.
Bretscher, M.S., 1971, *J. Mol. Biol.* 58, 775.
Chamberg, G.C., P. Häyry and L.C. Anderson, 1976, *J. Cell Biol.* 68, 642.
Cullen, S.E. and B.D. Schwartz, 1977, *J. Immunol.* 117, 136.
Dierich, M.P. and R.A. Reisfeld, 1975, *J. Immunol.* 114, 1976.
Laemmli, U.K., 1970, *Nature* 227, 680.