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Note

Isolation and purification of HLA-DR antigens from tumour cells by affinity chromatography and chromatofocusing

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The major histocompatibility complex (MHC) controls the expression of several membrane proteins in human and other mammals.

Owing to the high degree of polymorphism of these antigens, each organism can recognize its own proteins very specifically. This plays an essential role in the interactions of the immune system. The HLA-DR region of the MHC, localized in the short arm of the 6th chromosome controls antigen expression (Ia-like), whose importance in immunological reactions has been demonstrated [1—4]. Ia-like antigens are glycoproteins composed of two subunits of a molecular weight of 34,000 (α-chain) and 28,000 (β-chain). Their presence has been demonstrated in the cellular lineages of the immune system: B lymphocytes, T lymphocytes and macrophages [5]. This antigen can be expressed in the lactating mammary gland [6] and in certain malignant cells such as melanoma [7].

Apart from lactation, the normal mammary gland does not express this

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antigen. However, certain breast epithelioma may express Ia-like antigens. In fact, by analogy with the lactating gland, it would be interesting to relate the percentage of prolactin receptors on mammary tumours to the expression of Ia antigens on these same tumours. The setting up of such a relationship would involve a quantitative method of determining Ia protein on tumour cells. Until now, different published works have attempted to identify the protein, and have only led to the determination of its presence or absence on a given cell [8, 9].

The procedure of isolating the Ia-like antigen, which different authors have described [8, 9], consists of purifying membrane glycoproteins by affinity chromatography on lectin, immunoprecipitation with monoclonal antibody immune complex fixation on *Staphylococcus aureus* or protein A—Sepharose and protein release by immune complex dissociation. With this procedure, the antigen can be identified, but the proportion of glycoproteins it represents cannot be evaluated quantitatively.

To this purpose, a technique has been developed which combines the double labelling (^{131}I, ^{125}I) of membrane proteins and antibodies, and isolation of the immune complex by affinity chromatography and chromatofocusing. Thus a purified and doubly labelled immune complex has been obtained which allows a quantitative evaluation of the antigen linked to the antibody.

EXPERIMENTAL

Tumour cells

Cells were obtained by enzymatic dissociation [10]. Solid tumours are cut into thin strips then incubated at 37° C for 2 h in an RPMI 1640 medium containing 10% foetal calf serum, 0.4% collagenase I, and 0.002% deoxyribonuclease I. The number of viable cells is determined by trypan blue exclusion.

Products

Sephadex G-25, lentil-lectin—Sepharose 4B, gel PBE 9-4, and polybuffer 9—6 were from Pharmacia (Uppsala, Sweden), protein A—Ultrogel was from IBF (Villeneuve la Garenne, France), Nonidet P 40 from Serva (Heidelberg, F.R.G.), and glucose, glucose oxidase, and lactoperoxidase were from Boehringer (Mannheim, F.R.G.). The monoclonal human anti-Ia antibody produced in the mouse was from the Ortho-Pharmaceutical Corporation (Raritan, NJ, U.S.A.). ^{125}I and ^{131}I were from the Atomic Energy Commissariat (France). The Bolton and Hunter ^{125}I reagent was from Amersham (U.K.)

Cell labelling with ^{131}I

The cell suspension, at a concentration of 10^7 cells per ml in 0.05 *M* phosphate buffer pH 7.2 containing 0.15 *M* sodium chloride (PBS) was added to 10 units of lactoperoxidase, 10 units of glucose oxidase, 100 μg of glucose, and 1 mCi of $Na^{131}I$. After 10 min incubation at room temperature, the reaction was blocked by the addition of 50 μl of a tyrosine solution (0.5 *M*) in PBS.

Solubilization of membrane proteins

The suspension of labelled cells was centrifuged for 10 min at 600 *g*. The cells were then washed twice in PBS and suspended in 1 ml of PBS containing 2% Nonidet P 40, for 15 min at 4° C with gentle stirring.

The suspension was then centrifuged for 30 min at 30,000 *g*. The protein concentration was measured using the method of Lowry et al. [11]. Labelled proteins were purified on a small Sephadex G-25 column (5 cm × 1 cm), eluted with PBS.

Purification of glycoproteins on Lens culinaris lectin—Sepharose 4B column

The labelled proteins were placed at the top of a *Lens culinaris* lectin—Sepharose 4B column (10 × 0.9 cm) that had previously been equilibrated with 2—3 vols. of PBS.

The column was eluted at 15 ml/h with 30 ml of PBS, then with 15 ml of PBS containing 2% α-methylmannoside, which selectively desorbs glycoproteins. Fractions of 1 ml were collected by an automatic collector and counted in a dual-channel autogamma spectrometer. The fractions containing glycoproteins were concentrated up to a volume of 1 ml by ultrafiltration (Immersible C × 10, Millipore).

Antibody labelling with ^{125}I

The procedure of Bolton and Hunter was used [12]. First, 100 μCi of Bolton and Hunter reagent ^{125}I (2000 Ci/mmol) in 10 μl of benzene were evaporated under nitrogen in a small glass tube. Then 40 μl of a solution containing 80 μg of monoclonal anti-Ia antibody in PBS pH 7.2 were added. The reaction was left to take place for 10 min at 4° C, and was then blocked by adding 20 μl of 0.05 *M* borate buffer pH 8.2 containing 0.5 *M* glycine. The labelled antibody was purified by chromatography on a small column (3 × 0.5 cm) of Sephadex G-25. Its specific activity was 0.2—0.4 μCi/μg.

Immmunoprecipitation

The solution of ^{131}I-labelled proteins obtained as previously described was incubated at room temperature by stirring with 80 μg of ^{125}I-labelled antibodies.

Isolation of immune complex on protein A—Ultrogel

The reaction medium was fixed on to a protein A—Ultrogel (6 × 0.9 cm) column previously equilibrated with 2—3 vols. of PBS. The column was eluted at 15 ml/h with 20 ml of PBS, then with 10 ml of 0.025 *M* glycine buffer pH 2.6 which desorbed the doubly labelled immune complex.

Purification of immune complex by chromatofocusing

The fractions containing the immune complex previously dialysed against 0.025 *M* ethanolamine buffer pH 9.4 were fixed on the top of a PBE 9-4 gel column (30 × 0.9 cm) equilibrated with 0.025 *M* ethanolamine buffer pH 9.4. The elution was conducted at 10 ml/h with 150 ml of polybuffer 9—6 pH 6. Fractions of 2 ml were collected with an automatic fraction collector and counted in a dual-channel autogramma spectrometer.

RESULTS AND DISCUSSION

The solubilization of cellular membranes yielded 2—4 mg of labelled proteins per 10^7 cells. The specific activity of these proteins was 20—40 μCi/mg. The extraction yield of glycoproteins on *Lens culinaris* lectin—Sepharose 4B was 2—4% in agreement with results obtained on T lymphocytes [9]. The immunoprecipitation of the Ia glycoprotein by monoclonal antibody led to the formation of an immune complex that could not be isolated quantitatively by ultracentrifugation.

In these conditions, the method of choice for isolating the immune complex is affinity chromatography on protein A, a ligand specific to immunoglobulins. Fig. 1 shows the elution pattern obtained by chromatography on protein A—Ultrogel of the reaction medium after immunoprecipitation of ^{131}I-labelled glycoproteins with monoclonal antibody labelled with ^{125}I. The PBS buffer eluted only the ^{131}I proteins, while the glycine buffer pH 2.6 eluted doubly labelled proteins. This result shows that all of the immune complex was bound to protein A. The elution yield of ^{125}I was 85%; 8—12% of glycoproteins were eluted simultaneously with the antibody. In order to test the possible non-specific link to protein A—Ultrogel, labelled membrane glycoproteins underwent chromatography in the same conditions; 85—90% of the proteins were eluted by the PBS buffer, and 2—6% by the glycine buffer pH 2.6. This shows that some glycoproteins can be linked to protein A—Ultrogel at pH 7.2, and released at an acidic pH. It is obvious that the immune complex eluted by the glycine buffer is greatly contaminated by proteins that are not specifically linked to the gel.

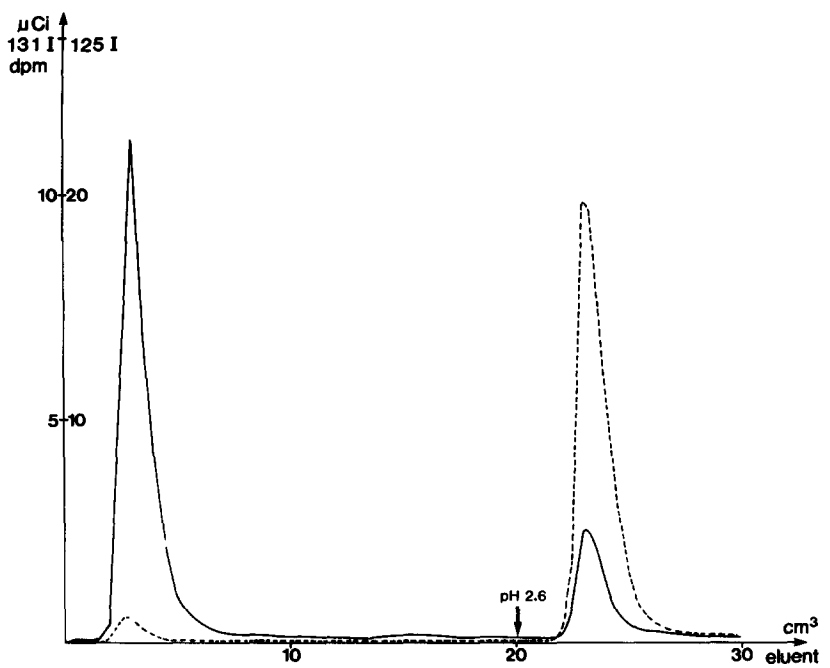

Fig. 1. Separation of the Ia—anti-Ia immune complex from solubilized membrane glycoproteins on protein A—Ultrogel column. Eluent 1: PBS pH 7.2. Eluent 2: 0.025 *M* glycine buffer pH 2.6. (——), ^{131}I; (- - - -), ^{125}I.

The final purification method of the immune complex was chromatofocusing. Indeed, antigen—antibody reactions lead to the polymerization of proteins reaching very high molecular weights, which inhibit gel filtration and polyacrylamide gel electrophoresis. A method that used the separation of proteins as a function of their isoelectric point, irrespective of their molecular weight, appeared to lead to a satisfactory purification of the immune complex. The isoelectric chromatofocusing consists of eluting an anionic ion-exchange column equilibrated at an alkali pH with a buffer containing an ampholyte mixture (polybuffer) with an acidic pH. During elution, a pH gradient will form between the pH of the gel and the buffer pH. Since the isoelectric point of γ-globulins falls between 7.3 and 6.3 [13], a pH gradient of 9 to 6 (polybuffer 9—6) was chosen. The protein solution was poured on to the column with a pH of 9.4. The negatively charged proteins were fixed on to the gel. They will be selectively released each time the pH gradient reaches the pH of each protein. Thus the proteins are focalised in a very small volume of eluent, which makes very fine separation possible.

Fig. 2 shows an example of the purification of the immune complex isolated on protein A—Ultrogel. The fraction containing the antibody was eluted at a very narrow peak, at pH 7.15. The other labelled fractions only contained ^{131}I. The purity of the doubly labelled immune complex was tested as follows: the glycoproteins were incubated with the ^{125}I-labelled antibody in the presence of 10^6 T lymphocytes. The T lymphocytes contain the Ia antigen in their membrane proteins. If this protein is specifically linked to the antibody, the

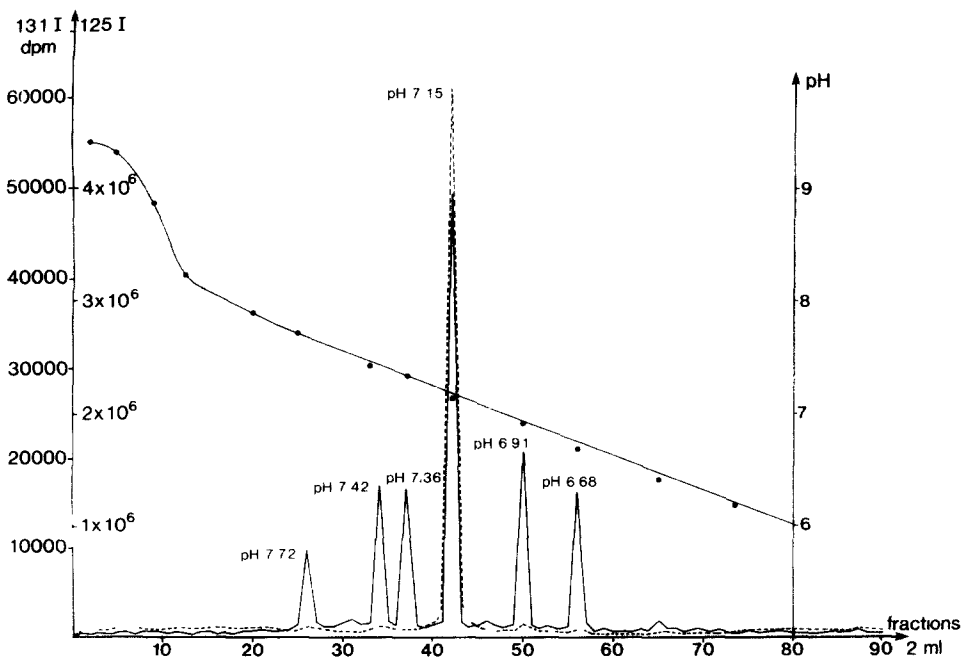

Fig. 2. Elution pattern of doubly labelled immune complex obtained from breast tumour cells on PBE 9.4 gel column, eluted at a flow-rate of 10 ml/h with polybuffer 9—6 pH 6. (——), ^{131}I; (- - - -), ^{125}I; (•——•), eluent pH.

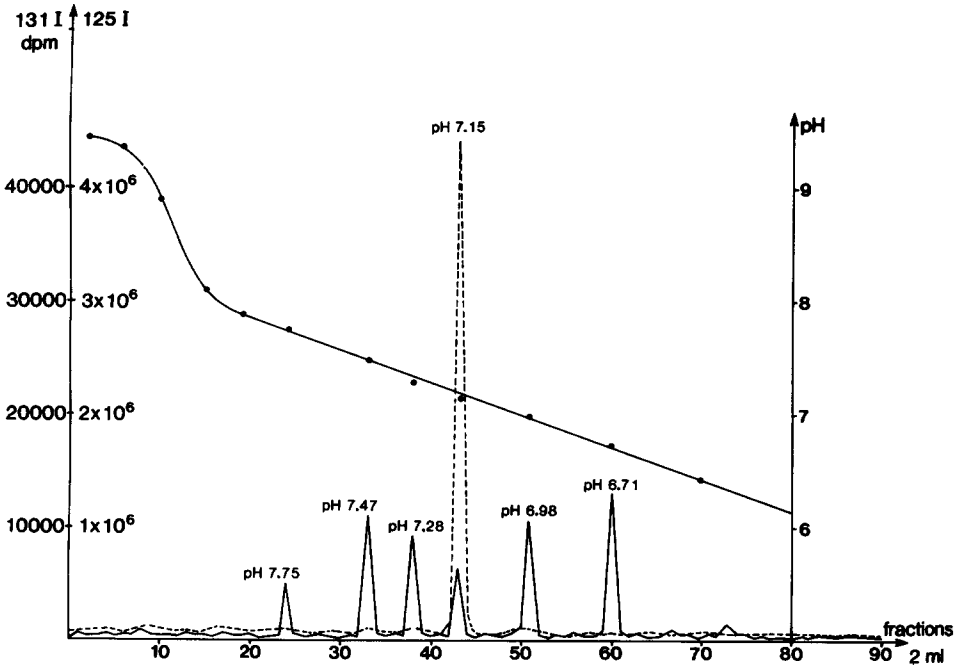

Fig. 3. Elution pattern of doubly labelled immune complex obtained from breast tumour cells after displacement of the equilibrium by leukemic lymphoid cells. Other conditions as in Fig. 2.

TABLE I

PURIFICATION OF THE Ia ANTIGEN—ANTI-Ia IMMUNE COMPLEX FROM 10^7 TUMOUR CELLS

Results are expressed as μg of protein.

	Labelling and solubilization	*Lens culinaris* lectin—Sepharose 4B	Protein A—Ultrogel	Chromatofocusing
^{131}I-labelled proteins	2000—4000	60—200	5—24	1.8—6
^{125}I-labelled antibody	80		68	54

equilibrium of the reaction of ^{131}I-labelled antigen with ^{125}I-labelled antibody must be displaced in the presence of the non-labelled antigen, and, consequently, the amount of ^{131}I linked to the antibody must decrease. Fig. 3 shows that in the presence of lymphocytes the doubly labelled eluted fraction at pH 7.2 had its ^{131}I activity markedly decreased, while the activity of the other ^{131}I-labelled fractions was not modified. These results show that the doubly labelled fraction contained the Ia—antibody immune complex. Since the specific activity of the ^{131}I-labelled proteins is known, the mass of Ia antigen linked to the antibody can be deduced. Table I shows the results of the purification of the Ia

antigen from solubilized membrane proteins; 1.8—6 μg of Ia were obtained in this way from 2000—4000 μg of protein. The antibody yield was 85% after chromatography on protein A—Ultrogel, and 67% after chromatofocusing.

Thus, a method to measure the amount of Ia antigen present in membrane proteins is available for mammary tumour cells. This may provide a way to evaluate the extent to which the expression of this antigen is a function of hormonal induction.

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