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An Immunoenzymatic Method for Improving the Sensitivity of Antigen Measurement by Electroimmunodiffusion Techniques. Application to the Quantification of Human α-Fetoprotein

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A double antibody technique of electroimmunodiffusion, which uses glucose oxidase-labelled sheep antibodies to rabbit immunoglobulins as second antibody, is described. Primary antigen-antibody reaction is carried out with a rabbit antiserum by electroimmunodiffusion.

The glucose oxidase-labelled immunoreagent, being of general application, can serve for the quantification of different antigens and is here used for measurement of low levels of human alpha-fetoprotein.

Reproducible results in the range of 50-800 ng/ml were obtained with a variation coefficient of 5 to 10 %.

Radioimmunoassay (RIA) is a very sensitive technique widely used for antigen measurements. It is, however, a complex technique requiring radioisotopes and and expensive laboratory equipment. To avoid this difficulty quantitative methods of gel immunodiffusion (6, 8) can be used as an alternative, although they are much less sensitive than RIA and for this reason of limited application. Immunoenzymatic techniques to enhance the visualization of gel immunoprecipitates have been recently reported (5, 15).

In the present paper a double antibody technique of electroimmunodiffusion is described using glucose oxidase labelled sheep antibodies to rabbit immunoglobulins as second antibody. Primary antigen-antibody reaction is carried out with a rabbit antiserum by electroimmunodiffusion.

The glucose oxidase labelled immunoreagent is of general application and can

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be used for the quantification of different antigens. In this paper the method is applied to measurement of low levels of human alpha-fetoprotein (AFP).

Materials and Methods

Immunochemicals. Anti-AFP serum * was prepared by immunisation of rabbits with semipurified samples of human AFP obtained from sera of patients with hepatocellular carcinoma, or from human fetal serum. Monospecificity was achieved by immunoadsorption with normal human plasma insolubilised by the method of AVRAMEAS and TERNYNCK (3).

Sheep antibodies to rabbit IgG were isolated from a commercial antiserum (Sheep anti-rabbit IgG, Antibodies Inc., Davis, Ca., USA) by immunoadsorption with rabbit IgG insolubilized on CNBr-activated Sepharose-4B (Pharmacia, Uppsala). Antibodies were eluted according to AVRAMEAS and TERNYNCK (3).

Glucose oxidase labelled immunoreagent. Isolated sheep antibodies were coupled to glucose oxidase (specific activity 210 u/mg, Boehringer, Mannheim) by the glutaraldehyde method of AVRAMEAS (2). After dialysis against phosphate buffered saline (PBS), 2 % of bovine albumin was added. The labelled antibodies (1 mg/ml) were distributed into 1 ml aliquots and lyophilized. The immunoreagent is stable for at least one year at room temperature.

Before use, the original antibody concentration (1 mg/ml) was reconstituted by adding 1 ml of water to the lyophilized aliquot and the solution was stored at 4° C. In this form the immunoreagent remains stable for several weeks.

Electroimmunodiffusion (6) (1st antigen-antibody reaction). A glass slide (9 × 12 cm) was precoated with 1 % agarose in water and dried. Melted agarose solution, 1 % in 0.05 M veronal buffer, pH 8.2, brought to 45-48° C, was carefully mixed at this temperature with the appropriate quantity * of anti-AFP serum and poured on the precoated glass slide to make a layer 2 mm thick. A series of 15 wells, 3 mm diameter, were cut in the gel to which were applied 10 μl samples of either AFP standards (generously provided by Dr. Uriel, Villejuif, France), or sera from patients. Hyperlipaemic sera were previously rendered free of lipoproteins by precipitation with dextran sulphate: 10 μl of 5 % dextran sulphate were added to 1 ml of serum, centrifuged at 800 × *g* for 10 min. and the supernatant recovered. The electrophoretic run was performed for 4 hours at 4° C and 110 V.

Sensitisation of immunoprecipitates (2nd antigen-antibody reaction). After electroimmunodiffusion the slide was washed overnight with mild stirring in a large volume of PBS and then soaked in a small volume (10-15 ml) of glucose oxidase labelled immunoreagent, the latter being adjusted immediately before use to an antibody concentration of 5 μg/ml by dilution with 0.5 % of bovine serum albumin in PBS. After 3 hours incubation at room temperature the slide was transferred to a moist chamber and kept overnight. Excess labelled antibody was removed by washing with several changes of PBS over 24 hours.

Staining of glucose oxidase labelled immunoprecipitate. The slide was first dried under filter paper. Colour development was carried out following the procedure of AVRAMEAS (2). The colour reagent was prepared by adding to 10 ml of 0.1 M potassium phosphate buffer, pH 6.8, 150 mg glucose, 5.0 mg MTT-tetrazolium salt

* Rabbit antiserum to α-fetoprotein obtained from Instituto ULTA (Zaragoza, Spain).

* With the antiserum used a final dilution of 1/1500 was optimal.

(Sigma) and 3.5 mg phenazine methosulphate (Sigma). The slide was incubated in the dark for approximately one hour. The immunoprecipitates stain blue-violet over a fainter background of similar colour. After staining the slide was washed under running water and dried.

Results and discussion

An example of the application of the method described to the quantitative determination of human AFP is shown in figures 1 and 2. Human AFP is a transitory liver antigen (16) characteristic of fetal life, whose serum levels decrease rapidly after birth and become undetectable by conventional immunodiffusion methods. Later reappearance of raised AFP levels has been associated with the presence of primary liver cancer and embryonal carcinomas (1, 17) or some non-neoplastic liver diseases (10, 12, 17).

Figure 1 shows the sensitised electroimmunodiffusion patterns of serial dilutions of three different samples (A, B and C) of human AFP. Within certain limits (see below) the height of the peak is proportional to AFP concentration.

Figure 2 shows the average values of four independent measurements of peak height plotted against AFP concentration. The relationship is practically linear in the range 50-800 ng/ml of AFP. Precipitates are still visible below 50 ng/ml but the variation coefficient is greater than 20 %. In the range 50-100 ng/ml the coefficient is reduced to 10 %, and to only 5 % between 100 and 800 ng/ml. Above 800 ng/ml, the relationship is not linear, but AFP quantitation at these higher concentrations can be carried out by conventional immunodiffusion methods (lower limit of sensitivity about 1 µg/ml). In practice, before attempting quantification by the enzyme-labelled method, it is preferable

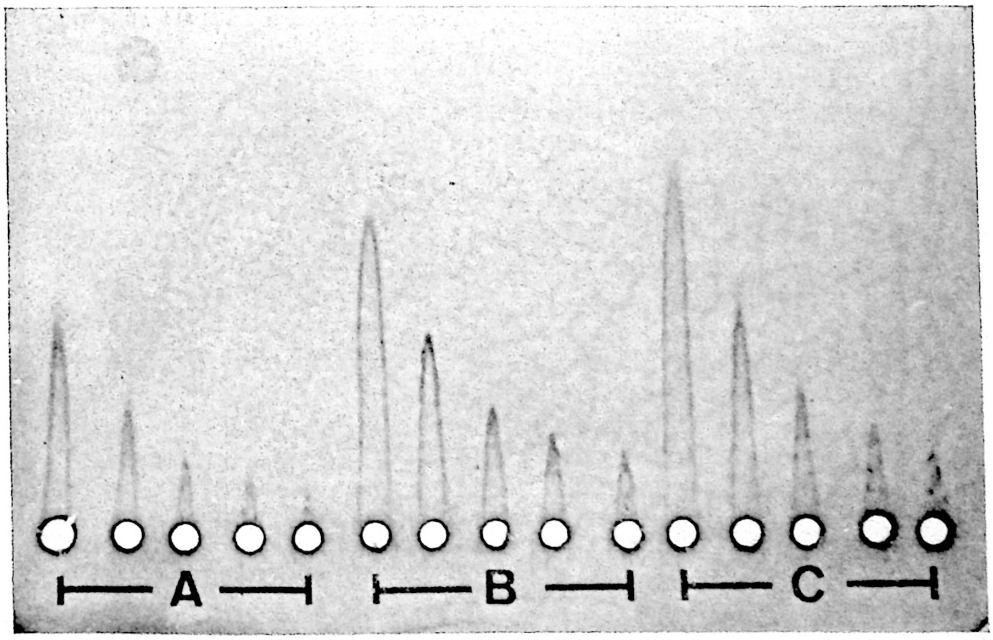

Fig. 1. *Electroimmunodiffusion patterns of serial dilutions (1/25, 1/50, 1/100, 1/150, 1/200) of three different samples A, B and C, containing 10, 18 and 21 µg/ml of AFP respectively.*

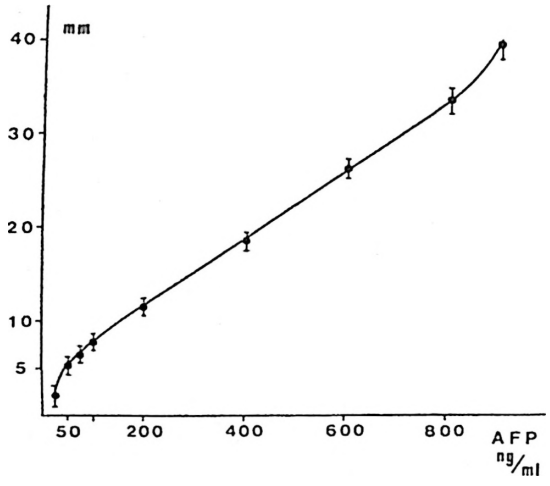

Fig. 2. *Standard curve of AFP concentration versus height of electroimmunodiffusion peak.* Each point represents average values of four independent measurements. Vertical bars show standard deviation.

to perform a conventional Ouchterlony test in order to check the concentration level of AFP. Samples which are positive in the latter test should be diluted appropriately for sensitised quantification.

The sensitivity of the method is slightly greater than that described by GUESDON and AVRAMEAS (5) using a double antibody radial immunodiffusion method. In addition electroimmunodiffusion has the further advantage of being a more precise and less time consuming technique than radial immunodiffusion.

The RIA techniques for AFP measurements (9, 14) are more sensitive and permit determination of AFP levels of the order of a few ng/ml. These low AFP concentrations are characteristic of human normal sera, hence for clinical purposes values of serum AFP below 25 to 50 ng/ml, are, at present, without pathological significance (4, 13).

MAIOLINI *et al.* (7) have recently described a non-competitive immunoenzymatic method, with sensitivity similar to that of RIA, which avoids some of the difficulties of the latter technique, but requires the use of purified preparations of AFP which are not readily available at present. On the other hand, the method described in this paper can be considered as a general technique and may be applied to other immunoprecipitable antigens provided that specific antisera are available.

The results of a study of AFP levels in human liver pathology using the method described here, will be presented elsewhere (11).

Resumen

Se describe un método de electroinmunodifusión con doble anticuerpo. La reacción primaria antígeno-anticuerpo se efectúa por electroinmunodifusión con un antisuero de conejo. Como segundo antisuero se utilizan anticuerpos de oveja, marcados con glucosa oxidasa, antiinmunoglobulinas de conejo.

El reactivo inmunoquímico marcado con glucosa oxidasa es de aplicación general y puede usarse para la determinación cuantitativa de antígenos diferentes. En este trabajo el método se aplica a la determinación cuantitativa de concentraciones bajas de alfa-fetoproteína humana.

Se obtienen resultados reproducibles entre 50-800 ng/ml con un coeficiente de variación del 5-10 %.

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References

1. ABELEV, G. I.: *Adv. Cancer. Res.*, **14**, 295-358, 1971.
2. AVRAMEAS, S.: *Immunochemistry*, **6**, 43-52, 1969.
3. AVRAMEAS, S. and TERNYNCK, T.: *Immunochemistry*, **6**, 53-66, 1969.
4. DELMONT, J., KERMAREC, J., LAFON, J., BONET, C., CASSUTO, J. P. and MASSEYEFF, R.: *Digestion*, **10**, 29-39, 1974.

5. GUESDON, J. L. and AVRAMEAS, S.: *Immunochemistry*, **11**, 595-598, 1974.
6. LAURELL, C. B.: *Anal. Biochem.*, **15**, 45-49, 1966.
7. MAIOLINI, R., FERRUA, B. and MASSEYEFF, R.: *J. Immunol. Methods*, **6**, 355-361, 1975.
8. MANCINI, G., CARBONARA, A. O. and HEREMANS, J. F.: *Immunochemistry*, **2**, 235-254, 1965.
9. MASSEYEFF, R., BONET, C., DROUET, J., SUDAKA, P. and LALANNE, C.: *Digestion*, **10**, 17-28, 1974.
10. MUÑOZ, J. R., GUILLÉN, G., GÓMEZ-PUCH, L., GARCÍA-JULIÁN, G. and ARRAIZA, A.: *Med. Clin.*, **60**, 212-219, 1973.
11. MUÑOZ, J. R., PIÑEIRO, A., NAVARRO, M.ª L., PERDICES, A. and URIEL, J.: Manuscript in preparation.
12. OPOLON, P., HIRSCH-MARIE, H., GATEAU, PH. and CAROLI, J.: *Ann. Med. Interne*, **124**, 883-888, 1973.
13. RUOSLAHTI, E., SALASPURO, M., PIHKO, H., ANDERSSON, L. and SEPPÄLÄ, M.: *Brit. Med. J.*, **2**, 527-529, 1974.
14. RUOSLAHTI, E. and SEPPÄLÄ, M.: *Int. J. Cancer*, **8**, 374-383, 1971.
15. STANISLAWSKI, M.: *C. r. Acad. Sci. Ser. D.*, **271**, 1452-1455, 1970.
16. URIEL, J.: *Pathol. Biol.*, **17**, 877-884, 1969.
17. URIEL, J. and DE NECHAUD, B.: *Ann. Inst. Pasteur*, **122**, 829-839, 1972.