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COUNTERFLOW IMMUNOBLOTTING

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UDC 616.633.962.4-097-078.73:543.545

KEY WORDS: isotachophoresis; electroendosmosis; immunoblotting; urinary proteins; immunoglobulin light chains

The immunoblotting (IB) method, in which proteins separated by polyacrylamide gel (PAG) electrophoresis are transferred to nitrocellulose membranes (NCM), and then developed consecutively by mono- or polyclonal antibodies and corresponding immunoenzyme conjugates, has become widely used to characterize antigens as well as monoclonal antibodies [6]. However, successive treatment of NCM with several immunoreagents and careful washing of the membranes after each treatment make IB a long and laborious method, requiring large quantities of immunoreagents, in which to immerse the NCM.

In this paper we show how it is possible to develop antigens adsorbed on NCM automatically by means of immunoreagents carried in the membrane by the flow of liquid. This form is created in NCM by isotachophoresis (ITP), i.e., electrophoresis in a heterogeneous buffer system possessing a common cation but different anions [5]. During ITP on porous membranes, an electroendosmotic counterflow (EEC) is created in them; the velocity of this counterflow in the zone of the anodal electrolyte exceeds the velocity of electrophoretic migration of any negatively charged protein, which will as a result be transferred by the flow of liquid toward the cathode [1, 2].

If ITP is carried out on NCM in which proteins separated by electrophoresis are first "imprinted," EEC will create what can be described as a conveyer belt in them, capable of carrying the immunoreagents required for successive detection of the antigens used through them. We have used this approach to detect and to determine the electrophoretic characteristics of immunoglobulin light chains (L_κ and L_λ) in human urine.

EXPERIMENTAL METHOD

Cellulose acetate membranes (CAM) of "Cellogel" type (from Chemetron, Italy), 17 cm long and from 1 to 4 cm wide, and NMC (Schleicheer und Schull, West Germany or Bio-Rad, USA) with pore diameter of 0.45 μ, were used. To develop mouse monoclonal antibodies (MCA) components or an experimental production batch of peroxidase-antiperoxidase complex from the Gabrichevskii Enzyme Research Institute, Ministry of Health of the RSFSR (Gor'kii), including goat antibodies to mouse IgG, mouse MCA to horseradish peroxidase, and peroxidase itself [3], were used. The residual protein-adsorbing capacity of the NCM was blocked with a 10% solution of the commercial preparation Bona (a milk mixture for infant feeding, made in Finland) on buffered physiological saline (BPS). Tris (base) and β-alanine, analytically pure, obtained from Serva, West Germany, were used for ITP.

Before analysis the samples of urine were filtered through filters with pore diameter of 0.22 μ (Millipore, France) and dialyzed against 0.06 M Tris-HCl, pH 6.7. As the standards for L_κ and L_λ we used lyophilized preparations of Bence-Jones (BJ) proteins of the corresponding types, generously provided by Professor E. V. Chernokhvostova, G. N. Gabrichevskii Research Institute of Enzymology, Ministry of Health of the RSFSR.

ITP of the urine was carried out by the method described previously [1, 2] on Cellogel CAM, using samples of 40-100 μl per centimeter width of the membrane, containing 30 μl of

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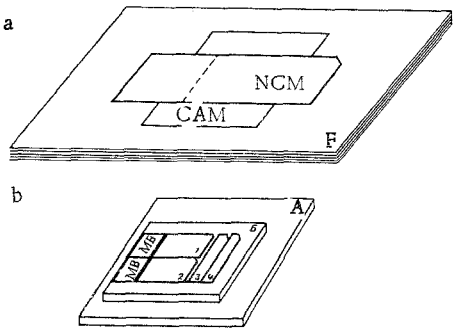

Fig. 1

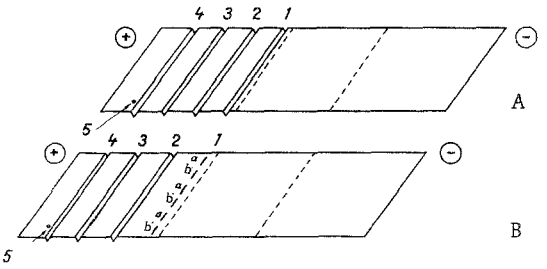

Fig. 2

Fig. 1. Diagram showing transfer of proteins to NCM. a) NCM prepared for blotting. Broken line indicates position of moving boundary (MB) during transfer of proteins from CAM and NCM. F) Pile of filter paper; b) A — Plexiglas support; B) slab of 10% PAG; 1, 2) CAM after electrophoresis; 3, 4) strips of CAM with BJ_K and BJ_λ.

Fig. 2. NCM prepared for automatic development. A: 1-4) Reservoir folds for reagents. Order of numbering corresponds to order of automatic processing of imprint with each reagent: 1) mouse MCA to human L_K, 1/20; 2) goat antiserum to mouse IgG, 1/4; 3) mouse MCA to peroxidase, 1/4; 4) peroxidase, 100 μg/ml; 5) vitamin B_{12} (reference). Broken line surrounds zone of development corresponding to Fig. 3a; B: 1) groove reservoirs for mouse MCA: a) to human L_K; b) L_λ (1/20); 2-5) the same as in A. Zone of reaction corresponding to Fig. 3b surrounded by broken line.

a 2.5% solution of ampholines (LKB, Sweden), pH 3.5-10.0.

Mouse MCA to constant regions of human L_K and L_λ-chains were generously provided by Professor O. V. Rokhlin (All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR).

To obtain an imprint, NCM 17 cm long and 1-10 cm wide, depending on the number of gels to be analyzed, were used. So that the protein-containing CAM was firmly in contact ("stuck") to the NCM, before blotting the latter were saturated with BPS. Excess of moisture was removed with filter paper, after which the NCM was placed on a wetted clean CAM lying on a pile of filter paper (Fig. 1a). The smooth surface of the CAM permitted uniform contact of the NCM lying on it with the membrane used for electrophoresis. The pile of filter paper consisted of three top sheets, wetted in BPS, and two bottom sheets, which were dry, and which rested on firm cardboard. The whole system was covered by a lid, which created a humid chamber.

A layer of 10% PAG 2 mm thick, made up in BPS, was spread out on a smooth Plexiglas support. On its top surface was laid strips of CAM with ITP-separated proteins: CAM with membranes used for electrophoresis of urine parallel to each other, and two strips of CAM, one of which was soaked in a solution of BJ_K and the other with BJ_λ in a concentration of 0.5 mg/ml, perpendicularly to their cathodal ends (Fig. 1b). The support with PAG was turned over with the membrane side downward and laid on the NCM so that no air bubbles were present between CAM and NCM. The whole system was placed under a vacuum in a gel drying apparatus (Bio-Rad, USA) at room temperature for 30 min. Under these circumstances part of the fluid from the gel passed through the strips of CAM and carried proteins to the NCM, where they were firmly adsorbed. As a result, parallel imprints of electrophoretically separated urinary proteins were obtained on the NCM, with imprints of BJ_K and BJ_λ at their cathodal edge perpendicularly to them. The last two served as control of specificity of the antibodies and to verify that they were present in excess compared with antigens "imprinted" on the NCM.

After transfer of the proteins the NCM strip was placed in a 10% solution of Bona for 1 h, and then washed in BPS and 0.06 M Tris-HCl, pH 6.7, in the presence of traces of bromphenol blue. Next, reservoirs for reagents were formed on the anodal part of NCM, where there were no imprints (Fig. 2). For minimal volumes (from 1 to 6 μl) they were made in the

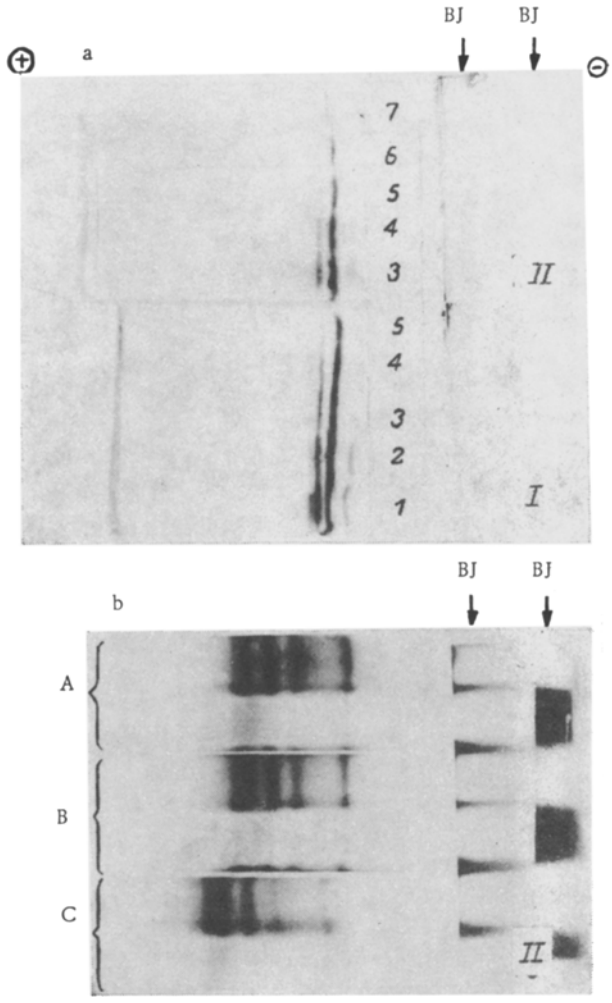

Fig. 3. Detection of immunoglobulin light chains by the IB method. a: 1-7) Immunoblotting of isotachophoresis gels of successive twofold dilutions of BJ_K: 1) contains 12 μg protein in 1 ml. Volume of each sample 20 μl/0.5 cm of CAM. Treatment as in Fig. 2A; b: A, B, C) immunoblotting of gels after isotachophoresis of urine from three normal individuals. Volume of sample 150 μl to 1 cm CAM. Treatment as in Fig. 2B.

form of folds from 0.1 to 1 cm deep, depending on the quality of reagent. The reservoirs were arranged at a distance apart so that the reagents in them passed through the blot consecutively, in an assigned order, completing and enhancing the action of the previous component, but not overtaking it. A distance of 15-20 mm satisfied these requirements.

For immunodevelopment of the "blot" ITP was used. For this purpose a strip of NCM was placed in the electrophoretic chamber with 0.06 M Tris-HCl, pH 6.7 (anodal buffer) and 0.012 M Tris-β-alanine, pH 8.6 (cathodal buffer) [1, 2]. The moving boundary (MB) was brought out with a voltage of 150 V. After stoppage of MB, complete dose of the corresponding reagent was introduced into all the reservoirs, and a spot of electrophoretically neutral reference substance, namely vitamin B_{12} (cyanocobalamin) was applied at the anodal edge of the reservoir with immunodevelopment closing reagent. The voltage was reduced to 25-20 V.

Under these conditions IB took place automatically through the night (15 h). If IB in the daytime was necessary, the whole electrophoresis was carried out under a voltage of 150 V in the course of 4-5 h.

The experiment was considered to be ended when the B_{12} spot reached MB. The reaction zone was cut out and immersed in developing solution: 18 mg 4-chlor-1-naphthol (Merck, West Germany), dissolved in 6 ml of ethanol, after which 24 ml of BPS, pH 7.4, and H_2O_2 up to a concentration of 0.01% were added to the mixture [4].

EXPERIMENTAL RESULTS

It must first be pointed out that the velocity of EEC in NCM is so high that MB reaches the stationary position close to the cathodal end of the strip of NCM in its last third, leaving the greater part of the membrane in the zone of most effective action of the counterflow.

Gels containing human BJ, obtained by electrophoresis and revealed by counterflow IB, are shown in Fig. 3a. The absence of background staining of the NCM will be noted. The reaction with L_K in the control zone is evidence that the developing reagents were used in sufficient excess and, consequently, that all components of the L_K family present in the preparation were detected. The sensitivity of the method in this case was about 2 ng protein per strip of NCM. Since about 100 µl of sample may be used in the experiment, it is possible by this method to analyze solutions even of a heterogeneous protein present in a concentration of 20-30 ng/ml.

Simultaneous demonstration of L_K and L_α in the urine of three normal individuals in the same strip of NCM is illustrated in Fig. 3b, where their heterogeneity can be clearly seen.

The counterflow IB method thus possesses high sensitivity and specificity and can be used to detect proteins on imprints of electrophoretic gels quickly and automatically. It is equally suitable for work with monoclonal and polyclonal antibodies and also with electrophoretic records obtained both on CAM and on PAG. The field of application of the method is very wide. It can evidently be used also for simultaneous determination of antibodies to different virus antigens in carriers of virus infections.

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METHOD OF DETERMINATION OF LIPID PEROXIDATION PRODUCTS IN BLOOD SERUM

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UDC 616.153.915-39-074

KEY WORDS: lipid peroxidation products; thermoluminescence; serum; wounds

The high specificity of biological membranes, the complexity and multiplicity of their lipid composition suggest that systems maintaining the lability of their composition and ensuring the rapidity of response of the cell to changes taking place in the body and also systems responsible for the strictly definite structural organization of membrane lipids, ensuring the effective performance of their biological functions, must coexist in membranes [2, 8]. One of the mechanisms of disturbance of this process and of injury to cells and intracellular organelles is lipid peroxidation (LPO) [2], which lies at the basis of many pathological states: burns [1], atherosclerosis and ischemic heart disease [5], aseptic inflammation [3], neoplastic diseases [2], etc.

Various methods are used to investigate LPO processes in biological systems: titrometric [6], spectrophotometric [10, 12], chemiluminescence [7, 9], biochemical [11, 13], etc. The main disadvantages of these methods are their laboriousness, the complexity and time-consuming nature of the investigations, their low level of accuracy, and their inadequate sensitivity.

The authors have developed a new, easier, and more sensitive thermoluminescence method of determining LPO products in blood serum, and a description of it is given below.

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Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 105, No. 5, pp. 628-630, May, 1988. Original article submitted July 17, 1987.