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TECHNICAL NOTE

Analysis of albumin charge by direct immunofixation in ultrathin gels

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The renal filter acts as a selective barrier towards size, charge and conformation of proteins, the last two factors being preeminent for molecules with a molecular radius greater than 36 Å [1]. Following the initial observation that large proteins with a cationic charge invariably have a higher renal clearance than their anionic homologes [2], studies have been performed to test the hypothesis that proteins with an altered charge may occur in vivo. By taking albumin as a reference marker, several attempts have been made to analyze albumin charge in biological fluids (serum, urines) in both experimental [3] and human [4–6] renal disease, but much work remains to be done. Technologies for analyzing albumin charge in biological fluids require the initial purification of the protein with a chromatographic technique (pseudoligand-chromatography on Affi-Gel Blue) [7] followed by analytical isoelectric focusing. Alternatively, an inverse strategy may be followed according to which albumin isoforms are fractionated by preparative isoelectric focusing in granulated gels, recovered from the gel by gel filtration and finally determined by monoclonal antibodies [5]. Though both methods give good results, owing to a few drawbacks their application in clinical studies has been so far quite limited. First of all, analytical isoelectric focusing of chromatographically purified albumin is indeed very sensitive, especially when new methods of protein staining with silver ions are used, but it is not quantitative. Secondly, some problems still exist with regards to the use of preparative isoelectric focusing followed by the direct immunologic determination by monoclonal antibodies of eluted isoforms since carrier ampholytes avidly bind albumin and induce a conformational rearrangement of the protein [8]. This variation may in turn modify the affinity of monoclonal antibodies for isoalbumins with a different charge and lead to artifactual results. Finally, the main drawback of both techniques which effectively limits their widespread application in clinical studies is related to the excessive length of execution. Recently, immunotechniques for direct immnofixation of proteins in agarose gel or after their transport to cellulose sheets (immunoblotting) have become the method of choice in analyzing in proteins in biological fluids. Immunoblotting techniques are, however, still time consuming, and immunofixation of albumin in agarose gels give a very broad resolution of the isoforms. Immunofixation of isoalbumins in matrices giving a good resolution, such as polyacrylamide, has never been attempted since large antibodies used for immunofixing albumin cannot penetrate traditional polyacrylamide gels. In this paper we describe a new technique for analyzing urinary albumin charge based on its direct immunofixation in polyacrylamide gels of extremely reduced thickness (up to 120 to 240 μm). In this condition (that is, reduced thickness of the gel) antialbumin antibodies are able to immunofix the protein to the gel without the need for transfer of the protein to other matrices.

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Methods

Patients

Seven normal children (mean age 7.4 ± 2.1 years) and nine insulin dependent diabetics (8 ± 3 years) were studied. Six IDDM children were normalbuminuric (UAE < 10 μg/min) while three others of the same group were microalbuminuric (UAE between 100 and 200 μg/min).

Preparation of the sample

In order to give a final content of albumin (approximately 20 μg), appropriate aliquots of urine were dialyzed against distilled water and lyophilized. Serums were adequately diluted to give the same concentration of protein.

Ultrathin isoelectric focusing

Isoelectric focusing was performed in ultrathin (120 to 240 μm) polyacrylamide slabs (total monomer concentration T = 7%; relative percentage of N,N′-methylenebisacrylamide C = 4%) supported by glass plates (25 × 12 cm) which were prepared in our laboratory and had been pretreated for a few minutes in 0.1% methacryloxypropyltrimethoxy silane (LKB, Bromma, Sweden) in acetone. Gaskets (25 × 12 cm) were formed by Parafilm rectangles, one layer giving approximately 120 μm which were put between a silane-treated glass plate and a plastic surface. The polymerization solution (7.5 ml) containing 12% (wt/vol) glycerol, 2.5% (vol/vol) carrier ampholytes (LKB) in a nonlinear range of pH, (70% pH 4 to 6, 15% pH 5 to 7, 15% pH 5 to 8), 20 μl of 25% (wt/vol) N,N,N′N′-tetramethylaethylendiamine, and 50 μl of 10% (wt/vol) ammonium persulfate was injected with a syringe between the glass and the

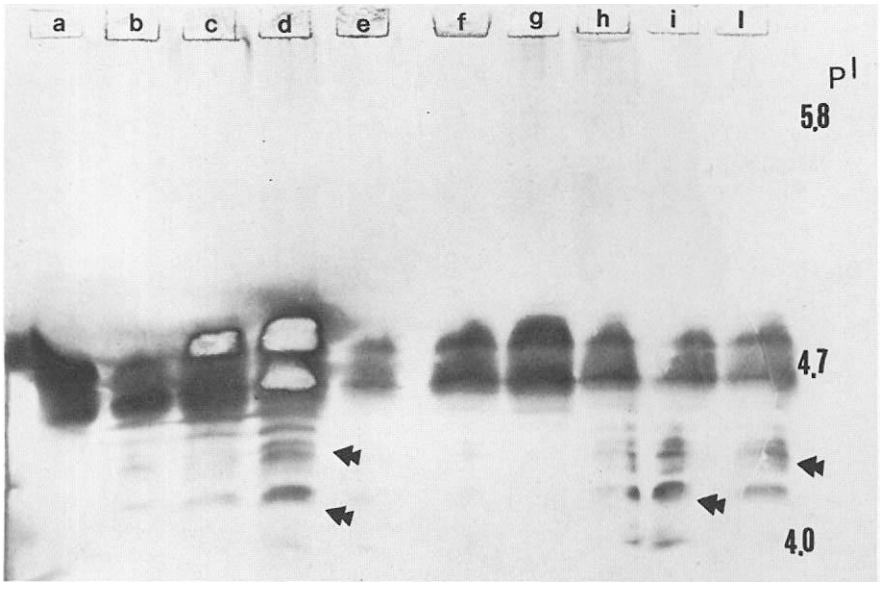

Fig. 1. *Ultrathin isoelectric focusing and direct immunofixation of urinary albumin from diabetic children.* Normal serum albumin (a), urinary albumin from normoalbuminuric (tracks b,c,d,h,i,l) and microalbuminuric children (tracks e,f,g). Twenty μg of albumin was applied to the gel. After electrophoresis, the protein was immunofixed by rabbit anit-human albumin antibodies and the complex was visualized with goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase.

plastic surface. Samples (50 μl) were applied with paper application pieces (LKB). Electrophoresis was performed at constant 10°C (using an LKB Multiphor system) with a pre-run of 500 V for one hour followed by 2000 V (13 watt) for six hours. The anode was 0.2 M H_3PO_4 and the cathode was 0.2 M NaOH. The pH gradient was determined with a surface glass electrode and/or by measuring the pH of the gel strips dissolved in 0.15 M KCl.

Immunofixation

Immediately after the electrophoretic run, polyacrylamide gels were covered with 7 ml of rabbit anti-human albumin (Dakopatts, Glostrup, Desmark) diluted (1:4) in normal saline and incubated for one hour in the dark. Extensive washings with 300 ml of normal saline were then carried out in the dark for 6 to 12 hours with four changes of the solution. The next step was to cover the gel with 7 ml of goat anti-rabbit Ig G antibodies conjugated with alkaline phosphatase (Bio Rad, Richmond, California, USA) diluted 1:500 in 20 mM Tris pH 7.5 containing 0.5 M NaCl, 0.05% Tween 20 and 1% (wt/vol) gelatin and incubated for two hours in the dark. After washings with 20 mM Tris pH 7.5 for a total of 15 minutes (with 3 changes) the violet color was developed by adding 0.03% Nitroblue Tetrazolium and 0.015% 5-Bromo-4 chloro-3 Indolyl phosphate in 0.1 M $NaHCO_3$ pH 9.8 containing 1 mM $MgCl_2$ in the dark at 37°C with gentle agitation for 30 minutes.

Traditional technique

Urinary albumin was purified by pseudo ligand-chromotography on Affi Gel Blue [7] and analyzed by analytical isoelectric focusing in ultrathin gels and silver staining as already described [6].

Immunoblotting

Isoelectric focusing prior to immunoblotting was performed in polyacrylamide gels 0.5 to 0.7 mm in thickness, which were made up of the same composition as described for immunofixation. The transfer of proteins to nitrocellulose (Trans. blot, Bio Rad) was achieved by a horizontal semidry apparatus TE 70 Semiphor (Hoefer, San Francisco, California, USA) using 0.025 M Tris/0.192 M glycine pH 8.6 with 0.1% SDS as a buffer, according to the method of Tavey and Baldo [10]. Transfer was conducted at 25 V for one hour. SDS was removed electrophoretically from the nitrocellulose sheet, using the same buffer without SDS and applying 25 V for 20 minutes. Ten ml of rabbit anti-human albumin antibodies (dil 1:50) were then incubated for one hour at 37% with the cellulose sheet and then the membrane was saturated with 3% BSA in 0.01 M Tris-barbiturate buffer pH 8.9. After discarding this solution, the cellulose sheet was washed three times for five minutes each with 100 ml of 20 mM Tris-Hcl pH 7.5 buffer containing 500 mM NaCl and 0.05% Tween 20. Ten ml of alkaline phosphatase linked goat anti-rabbit IgG (dil 1:500) were incubated for two hours at 37°C and washed two times with TTBS (100 ml). The color was developed in 0.1 M bicarbonate buffer (50 ml) pH 8.3 containing 1 ml of a solution of 70% DMF, 30 mg of Nitro Blue Tetrazolium and 15 mg of 5 bromo-4 chloro 3-indolyl phosphate.

Results and discussion

Albumin can be immunofixed in gels of polyacrylamide which are used for isoelectric focusing if the thickness of the matrix is reduced up to 120 to 240 μm. This task may be accomplished if two experimental conditions are fulfilled, namely if polymerization is performed using silanized glass plates for supports and if Parafilm is used as gasket for determining the gel thickness, one layer being approximately 120 μm. Figure 1 is an example of the application of the new technique for the analysis of urinary albumin deriving from diabetic children. As already known [4] in diabetics who are normoalbuminuric (tracks b,c,d,h,i,l), urinary albumin is microheterogeneous over a wide range of pIs from 4.1 to 4.7, while in microalbuminuric diabetics (tracks e,f,g), it presents a single band with pI 4.7 that is the pI of normal human serum albumin (track a). The presence of very anionic albumin isoforms in urine of the same normoalbuminuric diabetics studied by immunofixation and its absence in microalbuminuric ones was hereby confirmed with a traditional

Table 1. Comparison of direct immunofixation in polyacrylamide gels of reduced thickness with semidry immunoblotting

	Principle	Advantages	Disadvantages
Immunofixation	Cross-linking of the specific Ag by the 1st Ab and wash out of other proteins. Staining of the immunocomplex by the 2nd Ab.	1) Simplicity 2) Specificity (due to the absence of other proteins) 3) Rapidity 4) Better resolution 5) Decreased background	1) Cost (more 1st Ab must be used)
Blotting	Transport of the specific Ag and other proteins to cellulose. Staining of the immunocomplex (Ag and the 1st Ab) by the 2nd Ab. Other proteins are tightly linked to cellulose.		

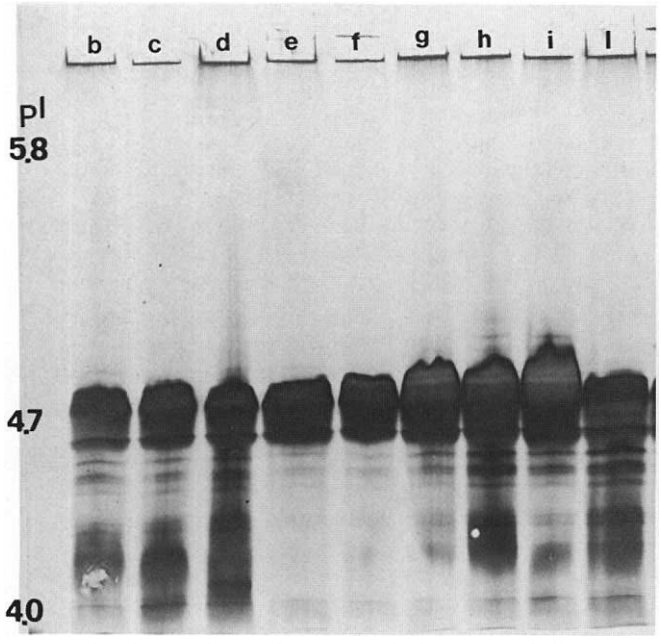

Fig. 2. *Ultrathin isoelectric focusing and silver stain of albumin purified by Affi Gel Blue from urine of the same normoalbuminuric (tracks a,b,c,h,i,l) and microalbuminuric diabetic children (tracks e,f,g) presented in Fig. 1.* After electrophoresis, albumin was stained with the silver method of Merril [9].

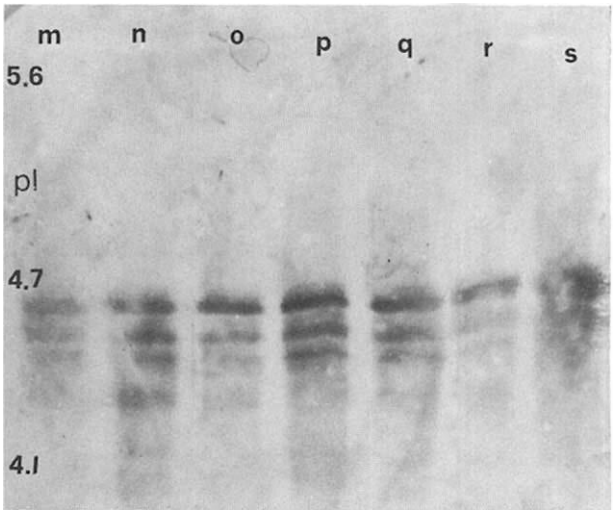

Fig. 3. *Semidry immunoblotting analysis of urinary albumin from 9 normal children.* The same amount of albumin (20 μg) was applied to gels for IEF and after the run were transblotted to nitrocellulose sheets as described [10].

technique of analyses based on the purification of albumin by pseudoligand chromatography followed by analytical isoelectric focusing (Fig. 2). While a clear separation and immunofixation of several isoforms of albumin is evident in gels of reduced thickness, a worse result was obtained by running the protein in gels of 500 and 1000 μm of thickness, in which case albumin was not immunofixed due to the difficulty of antialbumin antibodies in penetrating into the gel (not shown). A comparison of direct immunofixation with other immunoelectrophoretic techniques, such as semidry immunoblotting, is presented in Figure 3 and summarized in Table 1. In our experience, direct immunofixation gives better results compared to semidry immunoblotting in spite of the reduced time and difficulty of execution. Indeed, immunofixation is performed in three steps after isoelectric focusing (1st antibody, 2nd antibody, developing), while immunoblotting needs many more passages and two additional electrophoretic runs, one for transblotting the protein to cellulose and the other for removing SDS from the gel. A second advantage of direct immunofixation with respect to immunoblotting is that in immunofixation, the second antibody which brings the staining group reacts only with albumin, whereas other proteins are lost from the gel during the extensive washings. At variance, in immunoblotting all the proteins separated by isoelectric focusing are transblotted to cellulose and may in fact represent a potential site of reactivity with NBT, which is the chemical compound responsible for the staining [11]. Finally, due to the extreme thickness of gels used for immunofixation the separation of isoalbumins with different charges is better than in gels used for immunoblotting which cannot be cast on glass plates and must necessarily be thicker than 0.6 mm. In conclusion, a new method for analyzing albumin charge in urine is hereby described. By this method which is based on the direct immunofixation of the protein in

polyacrylamide gels of reduced thickness (120 to 240 μm), all urinary albumin isoforms were visualized in urine including the most anionic one that corresponds to the most glycosylated compound. With respect to traditional techniques for analyzing albumin charge and to immunoblotting, the new one is much quicker and easier, and should become the method of choice for studying the protein charge in urine in renal diseases.

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