Urinary excretion of glomerular basement membrane antigens in Alport's syndrome

A new diagnostic approach

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SUMMARY Alport's syndrome is defined by the combination of hereditary nephropathy and neurosensory deafness, and is diagnosed from the family history combined with renal electron microscopy. Immunoelectrophoresis of the urine of 8 of 12 children suspected of Alport's syndrome showed a precipitation line moving into the β -zone, applying an antiglomerular basement membrane antibody derived from an immunised rabbit. All patients who showed the typical pattern of Alport's syndrome on renal electron microscopy were among the 8 cases whose urine gave this immunoelectrophoresis pattern. Additionally, 5 of the mothers of the 8 children excreted the same antigen in their urine. The urine of 30 healthy children and of 10 patients with the idiopathic nephrotic syndrome did not show the presence of this antigen. This characteristic sign of Alport's syndrome may therefore be useful for its detection.

Alport's syndrome (AS) is a combination of hereditary nephropathy and neurosensory deafness (Marie, 1960; Perkoff, 1967). Additionally, abnormalities of the eye (Faggioni *et al.*, 1972), neurological problems (Mainzer *et al.*, 1970), and thrombopathy (Royer, 1975) may be present. Most important for the diagnosis are the history of the whole family and the clinical picture. Since the disease became known, attempts have been made to find a characteristic symptom or morphological pattern to enable early and precise diagnosis.

Foam cells in the interstitium of the glomeruli have been thought to be distinctive for AS (Goldbloom *et al.*, 1957; Whalen *et al.*, 1961), but the foam cells are not easily found in biopsy specimens and are not unique to AS (Krickstein *et al.*, 1966). The presence of small, fetal-like glomeruli has been considered helpful in diagnosis, but again they are not pathognomonic (Antonovych *et al.*, 1969). For the findings on light microscopy in AS Krickstein *et al.* (1966) have proposed the term 'mixed nephritis' to express the variable and uncharacteristic picture.

Specific alterations of the glomerular basement membrane (GBM) on electron microscopy were observed by several authors (Hinglais *et al.*, 1972;

Spear and Slusser, 1972; Churg and Sherman, 1973; Sessa *et al.*, 1974). Thickening, distortion, and transformation of the GBM into a network combined with electron lucent areas, containing sometimes small dark particles, was considered to be characteristic of AS. We ourselves found these changes in 6 of 12 patients (Fig. 1) (Balzar *et al.*, 1976).

Hill *et al.* (1974) however observed similar changes in other nephropathies such as post-streptococcal nephritis and focal segmental glomerulosclerosis, and concluded that none of the appearances described was diagnostic of AS. Nevertheless, these morphological changes, when combined with the characteristic clinical picture and family history, are likely to lead to a correct diagnosis of AS. Immunofluorescence studies show no specific deposits in hereditary nephropathy (Kaufman *et al.*, 1970; Hill *et al.*, 1974; Balzar *et al.*, 1976). In summary, in the absence of a full family history, early and exact diagnosis of AS is still difficult.

Considerations of pathogenesis based on heredity and a combination of renal, audiological, and ocular abnormalities led Spear (1973) to the conclusion that a defect common to the basement membranes might be caused by a defective composition of collagen. Evidence of abnormal constituents of the GBM in the urine of the patients with AS might therefore be helpful for the diagnosis of that disease.

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Fig. 1 Case 12. Electron micrograph of kidney biopsy. ($\times 14\,000$). BM = basement membrane: in the upper part, normal structure; between the arrows are thickening, distortion, and electron-lucent areas with dark inclusions. END = endothelial cell; EPI = epithelial cell.

Table 1 Clinical findings in patients with suspected Alport's syndrome (AS)

Case no.	Age (years)	Sex	Age of onset (years)	Haematuria	Proteinuria	Leucocytosis	Kidney function	Light microscopy	Electron microscopy	Eyes	Hearing defect	No. of affected family members*
1	17	Female	5	Yes, casts	Slight	Slight	Normal	Minimal proliferative glomerulonephritis	Not performed	Normal	R. ear	3
2	11	Female	14 m	Yes	"	"	Reduced	Refused		"	Normal	22
3	8	Female	14 m	"	"	No	Normal	"		"	"	22
4	9	Female	2½	Yes, casts	No	"	"	Minimal changes	AS	"	"	6
5	5	Male	4	Yes	"	"	"	Minimal changes	AS	Strabismus	"	6
6	10	Female	5	"	"	"	Reduced	Refused		Normal	Bilateral for high frequencies	3
7	13½	Female	3	"	Slight	Slight	"	Minimal proliferative glomerulonephritis	Not performed	"	Bilateral for low frequencies	3
8	11	Male	8	"	"	No	Normal	Minimal proliferative glomerulonephritis	AS	"	Bilateral for high frequencies	4
9	9	Male	3	"	"	"	Reduced	Minimal changes	Not performed	Strabismus	Normal	2
10	9	Male	6½	Casts	"	Slight	Normal	Minimal changes	AS	Normal	"	4
11	6	Male	4½	"	"	"	"	Minimal changes	AS	"	"	5
12	8	Female	8	"	No	No	"	Minimal changes	AS	"	"	5

*Interested readers may apply to the author for a duplicated copy of the family trees.

Patients, materials, and methods

Investigations of urinary excretion of GBM-antigens were made in 12 patients thought to have AS, details of whom are given in Table 1. Additionally, one family with hereditary nephrotic syndrome was examined (Table 2, XXVII-XXX), and one boy with noninherited chronic glomerulonephritis. As far as possible we tried to obtain urine samples of complete families. 30 healthy children and 10 children with the idiopathic nephrotic syndrome served as controls.

Preparation of antigen for immunisation. 8 normal human kidneys from persons aged between 20 and 40 years were obtained at necropsy, washed and perfused with cold phosphate-buffered saline (PBS), and comminuted carefully. This homogenate was forced through a 115 mesh sieve (metal) allowing the

glomeruli to pass, while adding several litres of cold PBS. A second filtration followed through a sieve (mesh 150) holding back the glomeruli. The glomeruli were put in 300 ml cold (4°C) PBS and collected by sedimentation in an MSE refrigerated centrifuge at $350 \times g$ for 10 minutes; the supernatant was discarded. The pellet was resuspended and sedimented four times. Aliquots of the suspension were sonicated in an ice bath by an MSE ultrasonic disintegrator, applying several bursts of 1 minute each of 1.6 A, 220 V at 5-minute intervals. The disrupted material was suspended in 1 M NaCl and centrifuged at $1400 \times g$ for 15 minutes. The GBM were washed three times in 1 M NaCl and three times in distilled water; the preparation was controlled by phase contrast microscopy and stored in the lyophilised state (Mahieu and Winand, 1970).

Table 2 *Immunoelectrophoresis and electron microscopy in patients with Alport's syndrome (AS) and their relatives*

Case no.	Sex	Haematuria	Electron microscopy	Mobility of GBM-antigen into β -zone	Reference no.
2	Female	Yes		Yes	I
3	Male	"		"	II
Mother		"		"	III
Father		No		No	IV
4	Female	Yes	AS	Yes	V
5	Male	"	AS	"	VI
Brother		"		"	VII
Mother		"		"	VIII
Father		No		No	IX
7	Female	Yes		"	X
	Male	Yes		"	XI
Father		No		"	XII
Mother		Yes		"	XIII
Grand-mother's mother		Yes		"	XIV
8	Male	"	AS	Yes	XV
Mother		"		Yes	XVI
9	Male	"		No	XVII
Mother		"		"	XVIII
Father		No		"	XIX
10	Male	Yes	AS	Yes	XX
Mother		"		No	XXI
Father		No		"	XXII
11	Male	Yes	AS	Yes	XXIII
12	Female	"	AS	"	XXIV
Mother		"		"	XXV
Father		No		No	XXVI
*B1	Male	Yes		"	XXVII
B2	Male	"		"	XXVIII
Father		No		"	XXIX
Mother		Yes		"	XXX
*C		Yes		"	XXXI

*Family B were cases of familial nephrotic syndrome.
Case C was a boy with chronic glomerulonephritis.

Immunisation schedule. At 2-week intervals rabbits were injected intracutaneously with 10 mg GBM mixed with aluminium hydroxide and complete Freund's adjuvant (Difco). 2 months later the animals were bled by cardiac puncture. The sera were absorbed eight times by glutaraldehyde insolubilised human plasma (Avrameas and Ternynck, 1969), platelets (twice), erythrocytes (three times), and white blood cells (three times). The absorbed sera were allowed to run against normal human serum in the double diffusion after Ouchterlony whereby no precipitation lines were found. Antibody activity against red blood cells was excluded applying a haemagglutination test and reactivity against platelets as well as white blood cells by agglutination assays. The anti-GBM-serum was tested by indirect immunofluorescence, producing a titre of 1:256 using human kidney obtained by biopsy. Absorption experiments as described by McPhaul and Dixon (1969) were made to investigate the relationship to the GBM.

Preparation of the patient's GBM antigen(s). The urine of patients was collected in plastic bottles containing 1% merthiolate. After ultrafiltration in an Amicon stirred cell (diaflo UF 2000) applying a membrane permeable for molecular weights below 20 000, they were dialysed against 0.1 M phosphate buffer of pH 7.4. Urines were concentrated 200 times and the protein content adjusted to a standard concentration of 20 mg/ml. Immunoelectrophoresis was performed on a LKB producter, at 50 mA and 340 V in 1% agar purum in a 1/15M veronal-sodium acetate buffer, pH 8.4. Electrophoresis was run for 50 minutes at room temperature. Diffusion took place during a 48-hour period and the precipitations photographed (Fig. 2a, b).

Fig. 2 Immuno-electrophoresis. (a) Precipitation lines of antiglomerular basement membrane serum, and concentrated dialysed urine of Case 11 (upper) and of his mother (lower) (electrophoresis XXIII and XXV, Table 2). An identical line is present in both. (b) The same line (below) from another patient with Alport's syndrome (Case 8, electrophoresis no. XV) compared with (upper) that from a patient with a different nephropathy (electrophoresis no. XXVII) which is located to the left, towards the α -zone.

Results

In 8 of our 12 patients with suspected AS (Table 2) we detected a precipitation line running in the β -zone of immuno-electrophoresis. The same line was found in 5 of their mothers, all of whom had permanent slight haematuria, and in one adult brother of Cases 4 and 5, who already had renal insufficiency. The fathers without haematuria never excreted this characteristic antigen. All the patients with the typical findings on renal electron microscopy were among the 8 cases with the characteristic immuno-electrophoresis. Family B (Table 2, immuno-electrophoresis XXVII-XXX) not finally diagnosed as suffering from AS, did not have the precipitation line in the β -zone but in the α_1 -region. Immuno-electrophoresis no. XXXI was from a boy with chronic glomerulonephritis; it showed a migration into the γ -fraction. The pattern observed in the patients with AS was not present in the group of healthy children, nor in the patients with the idiopathic nephrotic syndrome.

Discussion

Even healthy individuals excrete proteins into the urine (Sellers, 1956; Grant, 1959). Gilman (1935) described 'nonplasma proteins' in urine of patients with chronic glomerulonephritis and thought they derived from the kidney. Antoine (1960) and Antoine *et al.* (1962) showed that normal kidney antigens could be demonstrated in urine using anti-kidney serum; the phenomenon was intense in the nephropathy of potassium depletion. Excretion of renal tissue constituents was observed in nephrolithiasis (Boyce and King, 1963), in tubular disorders (Manual, 1963), and in toxic nephropathies (Intorp and Milgrom, 1968). Excretion of renal tissue constituents in lupus nephritis, acute glomerulonephritis, acute rejection of the kidney allograft, and tubular necrosis was shown by Antoine and Neveu (1968) who termed this 'histuria'. They applied antibodies 'specific to organ constituents', a water soluble kidney extract. Intorp and Milgrom (1968) described a thermostable kidney antigen and its

excretion into urine. They localised this antigen in the tubular apparatus and emphasised that the presence of the kidney-specific BE-antigen in the urine might facilitate the diagnosis of kidney diseases involving tubular damage.

In 1969, McPhaul and Dixon described immunoreactive GBM antigens in normal urine and serum. This led to the detection of soluble antigens in urine of healthy persons which were found to be immunologically related to GBM-antigens (McPhaul and Lerner, 1968). The antigens of normal individuals migrated in the postalbumin area in zone-electrophoresis, and these components cross-reacted with trypsin digests of homologous GBM. McPhaul and Lerner (1968) described in acute glomerulonephritis, antigens with γ -mobility and in chronic glomerulonephritis, antigens that moved with the albumin peak but with no regular pattern. The consistency of the pattern of precipitation lines seen in our patients with AS is therefore striking. The same patients were also found to excrete immunologically identifiable components of the GBM.

The electron microscopic (EM) picture varies greatly according to the degree of histological changes in the kidney. In general, the EM findings in AS are comparable with the changes seen in light microscopy, but are independent of age, sex, clinical symptoms, or duration of disease. The fact that 30 healthy individuals and 10 patients with the idiopathic nephrotic syndrome did not show the antigen described leads us to regard this protein as a pathological constituent of urine. It remains to be seen whether other nephropathies with the same antigenic pattern exist. We are at present studying the immunochemistry of AS.

Two families of Case 7 and of Case 9 in Fig. 2 who had formerly been classified as AS, did not show the precipitation line in the β -zone. Hereditary benign haematuria is the probable diagnosis in these cases. Both families suffer from a mild form of this condition and no deaths from uraemia are reported in them.

The method described will not replace other approaches to the diagnosis of this complex disease. However, once this pattern of urinary proteins has been found, in combination with the characteristic clinical and EM pictures, in one member of a family, further biopsies should be unnecessary. It remains to be seen whether this relatively simple method can be developed into a screening method for nephropathies.

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