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Acute Glomerulonephritis Associated with Streptococcus Pyogenes with Concomitant Spread of Streptococcus Constellatus in Four Rural Families

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ABSTRACT

We studied history, renal histopathology and microbiology of an epidemic of acute glomerulonephritis associated with throat infections and uncommon culture results in four neighbour families.

A 40-year-old man (index patient) was referred to a university hospital for dialysis and kidney biopsy due to a suspected acute glomerulonephritis. An acute tonsillitis had preceded the condition. Penicillin treatment had been started four days before the discovery of renal failure. Throat swabs were positive for β-hemolytic streptococci, group C (GCS). GCS were also found in throat cultures from his wife and two of their children. The bacteria were typed as *Streptococcus constellatus*. A third child had *S. constellatus* expressing Lancefield antigen group G. A neighbour and two of his children fell ill the following week with renal involvement. Throat swabs from both these children were positive for *S. constellatus*. His third child had erythema multiforme and *S. constellatus* in the throat while a fourth child had β-hemolytic streptococci group A; *Streptococcus pyogenes*. Kidney biopsies on the index patient and his neighbour showed an acute diffuse prolipherative glomerulonephritis compatible with acute post-streptococcal nephritis and microbiological analysis of renal tissue revealed in both cases *S. pyogenes and S. constellatus*. The

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families had had much contact and had consumed unpasteurized milk from our index patient's farm. In four of seven persons in two additional neighbouring families *S. constellatus* was found in throat swabs during the same month while two persons carried *Streptococcus anginosus* expressing the Lancefield C antigen.

In conclusion spread of *S. constellatus* coincided with the occurrence of four cases of acute glomerulonephritis. The two biopsied patients had both *S. pyogenes* and *S. constellatus* present in renal tissue. The epidemic either suggested that the outbreak of glomerulonephritis was due to *S. pyogenes* but coincided with the transmission and colonization of *S. constellatus* or that the *S. constellatus* strains were highly pathogenic or nephritogenic and that this organism can be transmitted in such cases.

INTRODUCTION

Streptococcus pyogenes or β-hemolytic streptococci group A (GAS) is recognized as the causative agent of tonsilllitis, impetigo, scarlet fever and septicemia and can induce glomerulonephritis and rheumatic fever as late complications [1, 2].

The relationship between acute glomerulonephritis and infections with GAS was first established in the early part of the 20th century, when Rammelkamp and coworkers showed that only certain strains of group A streptococci, especially type 12, were able to induce acute glomerulonephritis [3]. Cases or epidemics of acute glomerulonephritis are usually associated with pharyngeal infections or skin infections, i.e. impetigo [3, 4].

β-Hemolytic streptococci group C (GCS) can cause tonsillitis but very rarely glomerulonephritis [5, 6]. Group G streptococci have been considered to be less pathogenic than group C streptococci but have been associated both with glomerulonephritis and renal failure [7, 8].

Streptococcus constellatus, belonging to the "milleri" or "anginosus" group of streptococci sometimes express the Lancefield C-antigen. These bacteria differ from the "large colony type" GCS (*Streptococcus zooepidemicus*) in forming small, "pin point" colonies and having a different pathogenic potential. They belong to the normal flora of the mouth. They have not, to our knowledge, been associated with tonsillitis or glomerulonephritis but have been shown to occur in pharyngitis and peritonsillitis [9–11]. In this report we describe an epidemic of acute glomerulonephritis coinciding with the spread of *S. constellatus* in the patients families and in neighbouring families in a rural area of Sweden. Possible associations of the spread of the *S. constellatus* and the occurrence of four cases of acute glomerulonephritis, two of them biopsy-verified and one of them with a nephrotic syndrome, are discussed.

PATIENTS AND METHODS

Case reports:

A 40-year old man-the index patient (A)-was admitted to his local hospital, Eksjö, with signs of acute nephritis. Due to a high grade of uremia he was transferred the following day to the University Hospital of Linköping, Sweden for kidney biopsy and acute dialysis. Four days before arrival, he had started on a course of penicillin due to acute tonsillitis.

Three further patients (B-D) described below later developed clinical findings indicating an acute glomerulonephritis.

The acute renal failure in patient A remitted after treatment, transiently with cyclophosphamide, corticosteroids, plasmapheresis and dialysis. A kidney biopsy showed acute postinfectious glomerulonephritis.The patient was discharged from the hospital with a serum creatinine of 173 µmol/l (normal range 70-115 µmol/l). At the time of discharge a throat culture was positive for beta-hemolytic streptococci, Lancefield group C, reported as GCS. The same type of bacteria were also detected in throat cultures from the patient´s wife and two of their children. A third child had β-hemolytic streptococci Lancefield group G (GGS) in a culture from the throat while a fourth child was culture-negative.

Patient A´s renal function was subsequently normalized with both normal serum creatinine values and a normal iohexol clearance of 99 ml/min and 1.73 m^2 body square area in November 1997. There was no proteinuria but a slight hematuria (++) .

The week after the start of patient A´s illness a neighbour of our patient (B) fell ill (Figs 1 and 2). He was admitted to his local hospital, Eksjö, with renal failure (S-creatinine 200 µmol/l and urea 35 mmol/l, normal range 3-9 mmol/l). The neighbour had felt ill for about four weeks and had had a slight oedema. One week before

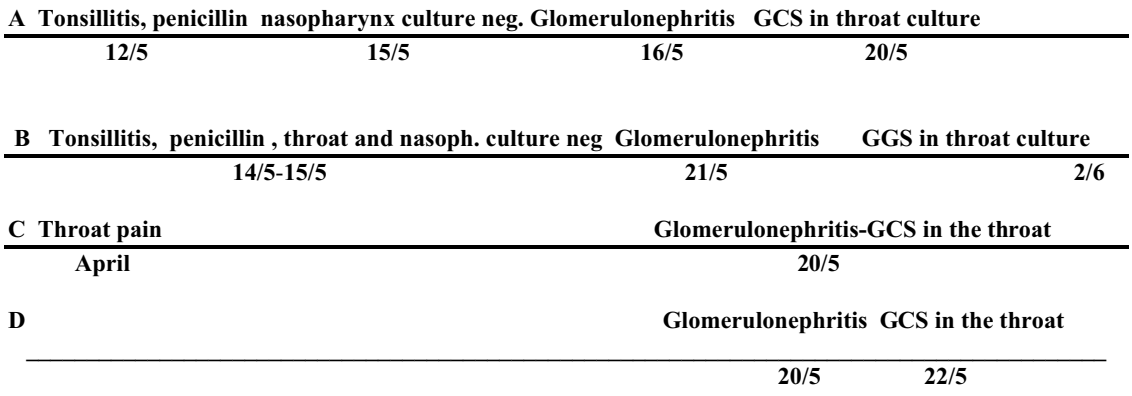

Fig 1. The time points of tonsillitis, throat cultures and glomerulonephritis in patients A–D.

admittance he had received a course of penicillin. A throat culture before the course was negative. Antistreptolysin O titre (ASO) was 200 (normally < 150) and anti-deoxyribonuclease B (ADNase B) titre was high, >1200 (normally < 400). A kidney biopsy was performed, see histopathology below. A throat culture 12 days afterwards was positive for GGS. His wife was healthy.

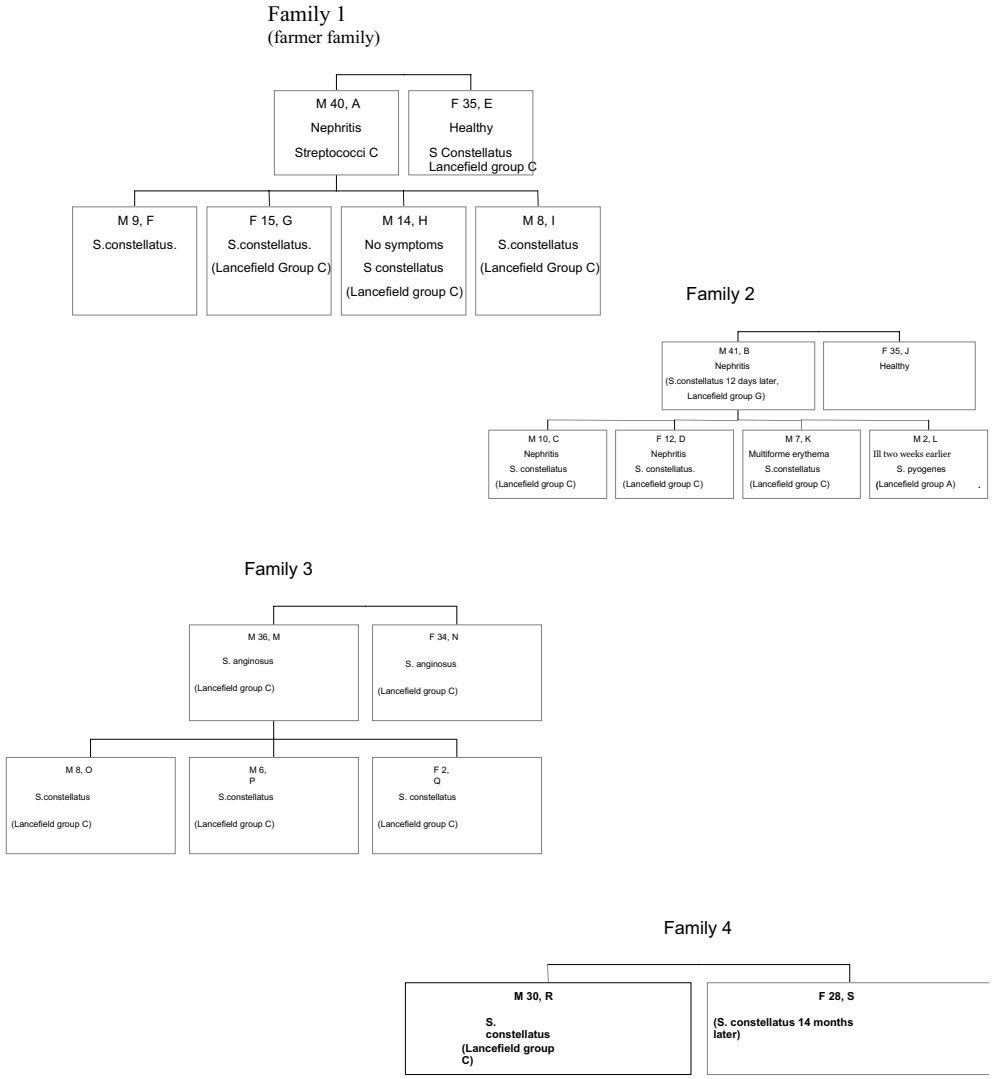

Fig 2. Schematic drawing of family 1 (farmer family). Schematic drawing of family 2 (neighbours). Schematic drawings of families 3 and 4 (neighbours).

Two of the neighbour´s children (C and D) were admitted to the central hospital of Ryhov in Jönköping. Both had fallen ill with renal involvement. The boy (C) had earlier had a sore throat. He had a hypertension with blood pressures of 140/80-160/120 mm Hg and was transiently treated with metoprolole. Creatinine was 71-83 µmol/l and urinary albumin 0.38 g/24 h (normally below 0.1 g/24h). Urinary sediment findings were pathologic. ASO was elevated to 560 and ADNase was also

high > 1200. Complement level C3 was low (0.14g/l), while C4 was normal (0.22 g/l). His twelve year old sister (D) had a nephrotic syndrome with urinary albumin levels of 3.3-9.7 g/l (normal < 0.03 g/l) and a serum albumin of 29-32 g/l (normal > 35 g/l). Serum creatinine was 69-98 µmol/l and the blood pressure was elevated to 150/90-150/100 mm Hg. In both children beta hemolytic streptococci, reported as GCS, were found in throat cultures. A third child who acquired erythema multiforme also had the same bacteria in the throat, while a forth child had group A streptococci (*Streptococcus pyogenes*). The time points of tonsillitis, throat cultures and onset of glomerulonephritis are shown in Fig. 1.

The families had had multiple contacts, and their children usually went to school by the same bus. The patient who first fell ill was a farmer, and the families had consumed unpasteurized milk from the farm of patient A. Cultures from the milk were, however, negative for GCS and other streptococci. In six of seven persons of two further neighbouring families, ß-hemolytic streptococci, reported as GCS, were found in throat swabs during the same month. Five of these persons were treated with penicillin. A throat culure from one of these patients 14 months later revealed beta-hemolytic streptococci group C.

Microbiology:

Throat swabs. Throat swabs were transported in Amies medium and cultured on 5% sheep blood agar containing Columbia agar base (Difco). β-hemolytic colonies

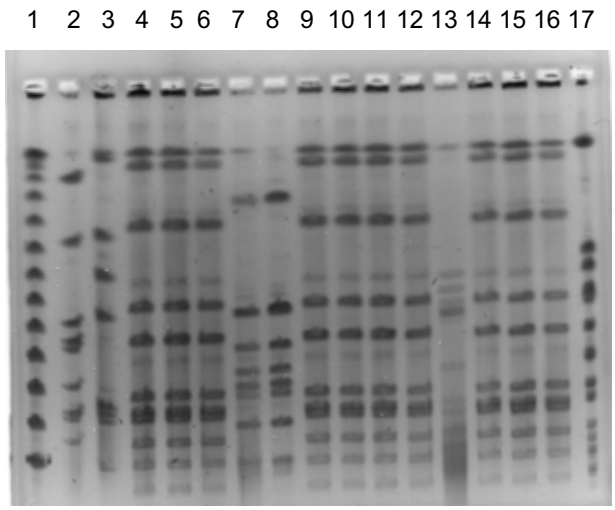

Fig 3. Pulse field gel electrophoresis of streptococci. Lane 1: DNA marker λ ladder. Lanes 2-16: patients DNA. Lane 17: DNA marker *S. aureus* 8325 (NCTC 8523).

were isolated and agglutinated with latex particles coated with antibodies to Lancefield group A, C and G antigens (Oxoid Ltd, Basingstoke, Hampshire, England).

Pulsed Field Gel Electrophoresis (PFGE). Chromosomal DNA from GBS embedded in agarose plugs was prepared as previously described [12]. An agarose slice was incubated overnight at 30°C with 20 U of *Sma*I, (MBI, Fermentas) in the provided enzyme buffer. Plugs were washed in TE-buffer (1h 5°C) before mounting into wells of a 1 % agarose gel (Pulsed Field Certified Agarose, Bio Rad) in 0.5x TBE-buffer pH 8.3. Electrophoresis was performed on an automated PFGE apparatus (Gene Path strain typing system, Bio Rad). Standard programs for fragment size 50-600 was used. A standard λ-ladder (New England Biolabs Inc, MA, USA) and SmaI digested *S. aureus* 8325 was included alongside the samples. The agarose gel was stained with 0.2% ethidiumbromide and washed in tap water visualized and photographed under UV light (Fig. 3).

PCR analysis of skaA

Five to ten colonies, picked from a blood agar plate, were resuspended in 50 ul sterile distilled water in a micro test tube and heated for 5 min at 100°C. After centrifugation, 10 ul of the supernatant was used as template in the PCR reactions. Reaction conditions were 0.2 mm dNTP´s, 20pmol of each primer with 0.5 U Taq polymerases buffer with 1.5 mM $MgCl_2$ (MBI Fermenta, Labassco) and bovine serum albumin (0.17 mg/ml). The amplification was performed on a PTC-200 thermal cycler (MJ Research, Scandinavian Diagnostic Services, (SDS) Falkenberg, Sweden) with annealing temperature of 55°C. Forward primer used was ska 453-477; 5´-AACCTTGCCGACCCAACCT-3´ and reverse primer ska 770-791; 5´-TCCATTGGTAAAATCGTACGG-3´ [13]. The PCR products were separated with electrophoresis on 0.7 % agarose gel.

Phenotypic typing of group C streptococci

Further typing of group C streptococci was performed with *API Strep* from BioMérieux sa Marcy l´Étoile, France.

Renal biopsy examination

Part of the renal biopsy material obtained from patients A and B was snap-frozen in isopentane-CO2 for immunfluorescence studies. Cryostate sections, 4-5 µm thick, were fixed in ethanol, incubated with FITC-conjugated rabbit antibodies to human IgG, IgA, IgM, and C3c (Dakopatts, Sweden), and examined in an incident-light fluorescence microscope. Another part of the biopsy material was immersed in Histochoice (Amresco, Solon, IL, USA), embedded in paraplast, and cut into 1- to 2-µm sections that were stained with periodic acid Schiff´ s reagent and with periodic acid silver methenamine. The remaining part of the biopsies was immersed in 2-% glutaraldehyde, embedded in Epon 812. Semi- and ultra-thin sections were prepared, stained with OsO4 and examined in a JEOL 1200 electron microscope.

Table 1: Pulse field gel electrophoresis – description of lanes in Fig 3.

Lane	Bacteria	Lancefield group	Patient
2	*S. constellatus*	G	B
3	"	G	F
4	*S. constellatus*	C	D
5	"	C	C
6	"	C	K
7	*S.anginosus*	C	M
8	"	C	N
9	*S.constellatus*	C	O
10	"	C	P
11	"	C	Q
12	"	C	R
13	" *	C	S
14	"	C	H
15	"	C	G
16	"	C	I
1	DNA marker, λ-ladder		
17	DNA-marker, *S. aureus* 8325		

*) isolated 14 months after the outbreak

Tissue preparation and lysis

A Nucleon kit [14] for the extraction of genomic DNA from tissue (Nucleon HT, product code RPN 8509), Amersham International plc & Scotlab Ltd 1997) was used. The tissue biopsies were grinded on liquid nitrogen to fine powder in a sterile mortar. Grinding occurred in a laminar air hood. Grinded tissue was transferred to an Eppendorf tube and 350 µl Reagent B was added. Thereafter 5 µl RNase solution was added to the tube and the contents were incubated for 30 min at 37°C. Proteinase K solution (18 µl) was added and the tissue was incubated at 56°C overnight. After centrifugation at 7,500 rpm for 5 min the supernatant was transferred to a new tube. For deproteinization 100 µl of sodium perchlorate solution was added and the content mixed by inverting the tube several times.

DNA extraction

Chloroform (600 µl) was added to the supernatant and the content mixed by inverting the Eppendorf tube several times. Nucleon resin (150 µl) was then added without mixing the content. Centrifugation occurred at 2,200 rpm for 1 min. The supernatant was transferred to a new tube and DNA was precipitated by adding 2 volumes (700 µl) of cold ethanol and by inverting the tube. The DNA was washed by centrifugation at 4,000 x g for 5 min. The supernatant was discarded and 1000 µl of 70% cold ethanol was added. After mixing, another high-speed centrifugation was made and the supernatant was discarded. The DNA pellet was dried in a vacuum evaporator for 15 min. Thereafter, the DNA was dissolved in TE buffer.

Checkerboard DNA-DNA hybridization

A slightly modified procedure of the checkerboard method developed by Socransky et al. [15] was used for DNA-DNA hybridization. Each DNA sample was pipetted in volumes of 100 µl and transferred to separate Eppendorf tubes. One hundred microliter of 0.5 M sodium hydroxide was added to the sample. All samples were placed in a water bath (100°C) and boiled for 5 min. They were then neutralized using 800 µl of 5 M ammonium acetate. The released DNAs from the clinical species and the type strains of *S. constellatus* NCDO 2226^T and *S. pyogenes* CCUG 4207^T were placed into the lanes of a Minislot (Immunetics, Cambridge, MA, USA) and then deposited on a nylon membrane (Boehringer Mannheim, Mannheim, Germany). The membranes were fixed by baking at 68°C for 30 min followed by exposure to ultraviolet light for 30 s. The membrane with the fixed DNA was placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90°C to the channels of the device. The membranes were prehybridized at 42°C for 1 h in 50% formamide (AppliChem, Darmstadt, Germany), 5 x SSC (1 x SSC = 150 mM sodium chloride, 15 mM sodium citrate (AppliChem), pH 7.0), 10% casein (Sigma, St. Louis, MO, USA), 5 x Denhardt's reagent (ApplicChem), 25 mM sodium phosphate (pH 6.5) and 10 mg/ml yeast RNA (Boehringer Mannheim). Digoxigenin-labeled, whole genomic probes and hybridization buffer containing 45% formamide (5 x SSC, 1 x Denhardt's reagent, 20 mM sodium phosphate (pH 6.5), and 10 mg/ml yeast RNA,

20 ng/ml labelled probe, 10% dextran sulphate (Sigma) and 10% casein were placed in individual lanes of the Miniblotter. The whole apparatus was covered with Saran wrap and transferred to a sealed plastic dish with water. The membranes were hybridized overnight with gentle shaking at 42°C. They were washed in a plastic dish to remove loosely bound probe. To detect hybrids, the membranes were blocked with 10% casein in maleate buffer (100 mM maleic acid, 150 mM sodium chloride, pH 7.5) and then incubated with antidioxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim), diluted 1:20 000 in maleic buffer. Signals were detected chromogenically using NTB/BCIP tablets (Boehringer Mannheim) overnight.

RESULTS

Microbiology

PFGE typing showed that 13/15 isolates reported as GCS were in fact *S. constellatus* and 2/15 were *S. anginosus* (Table 1 and Fig 3). Reagglutination confirmed that 13 of these 15 strains expressed the Lancefield C-antigen and that two belonged to group G. Eleven of the 13 *S.constellatus* strains expressed the C antigen. PFGE also showed that 10 of the 11 *S. constellatus* group C were identical (Fig 3, lane 4, 5, 6, 9, 10, 11, 12, 14, 15, 16). The eleventh strain of *S. constellatus* (Fig 3, lane 13) obtained from a throat culture 14 months after the outbreak, differed somewhat from the other 10. PCR-analysis of the *ska* gene showed that this gene was absent in all 15 isolates. The group C strain from the index patient (A) was not subtyped as it had been isolated in a different laboratory (Linköping) and was therefore not available for further analysis.

Epidemiology

The time points of tonsillitis and throat cultures are shown in Fig. 1.
The family trees and the results of the throat cultures with subtyping are shown in Fig 2.

Kidney histopathology

The kidney biopsy material from both patients A and B contained renal cortex with approximately 20 glomeruli. Histologically, a moderate to severe increase of glomerular endocapillary cells in a diffuse and global pattern was observed.

The proliferating endocapillary cells, together with infiltrating polymorphonuclear cells,encroached considerably on the capillary lumina (fig. 4a). In patient B occasional capillary loops contained a hyaline subendothelial material. There was no significant extra-capillary cell proliferation. Examination by immunfluorescence microscopy revealed in patient A large amounts of IgG and C3c, and moderate amounts of C1 q, outlining the glomerular capillary loops in a granular, "starry sky" pattern. Slight amounts of granular IgM and IgA deposits were also seen along the

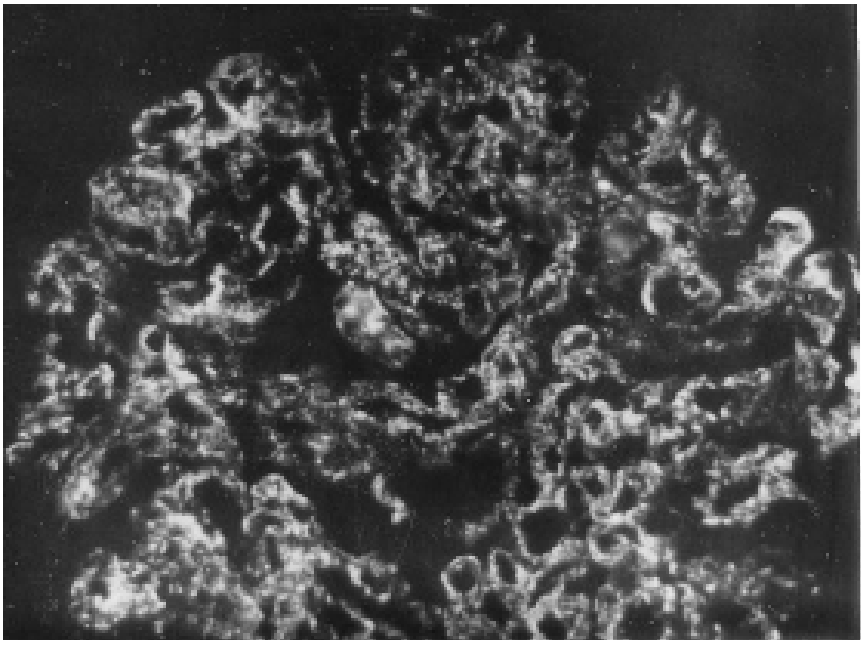

Fig 4a. Irregular deposits of C3c along the glomerular capillary loops and in the mesangial regions, so-called "starry sky pattern".

capillary loops. Patient B showed the same pattern of granular deposits with a dominance of C3c along the capillary loops (Fig. 4b), moderate amounts of IgG, and slight amounts of IgA, while there were no deposits of IgM and C1q. Both patients showed granular deposits of immunoglobulins and complement in the mesangial region. The ultrastructural examination confirmed presence of endocapillary cell hyperplasia, and electrone-dense subendothelial deposits. In some capillary loops in patient B, these electron-dense subendothelial deposits filled a large part of the cap-

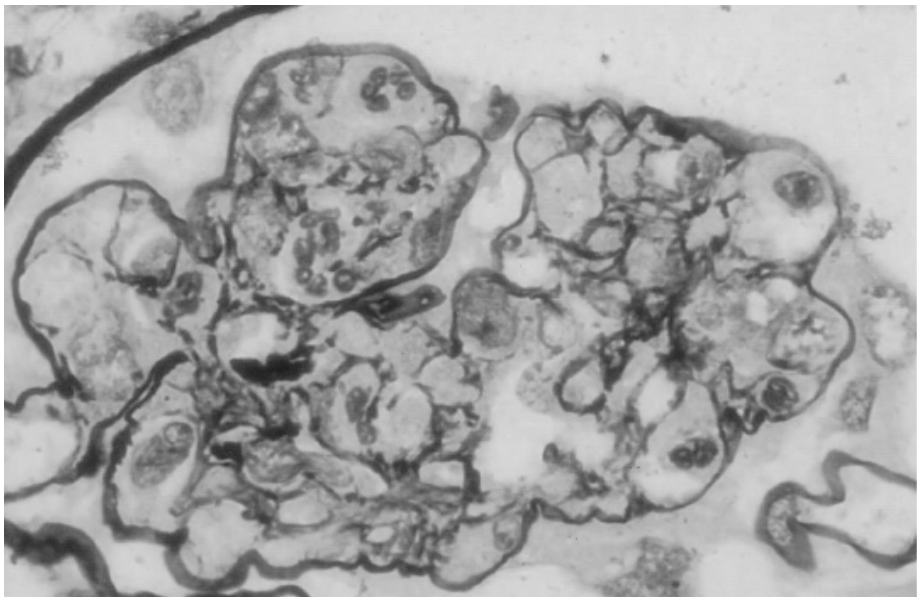

Fig 4b. Glomerulus with accentuated lobular configuration due to severe endocapillary cell proliferation. The proliferating cells together with infiltrating polymorphonuclear leukocytes fill out the capillary lumina.

illary lumen. Both patients had discrete, large, irregular subepithelial electron-dense deposits, so called “humps” (Fig. 4c). Patient A showed foci of tubulointerstitial inflammation sometimes with tubulitis.

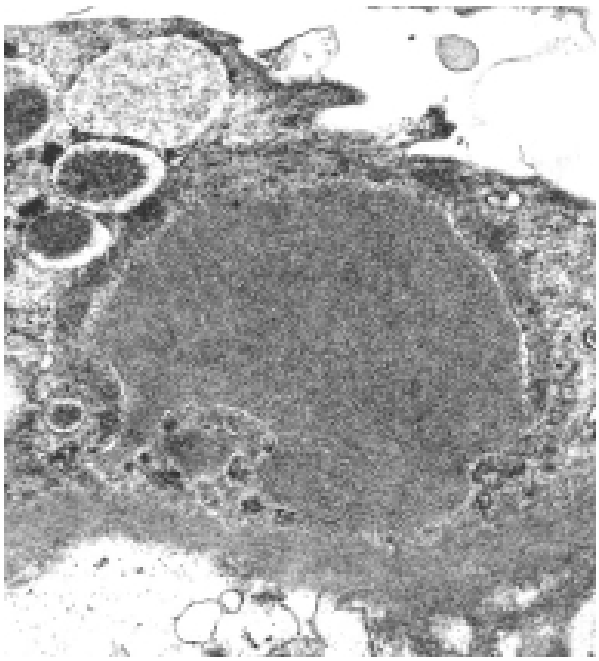

Fig 4c. Large, discrete electron-dense deposits on the subepithelial side of the glomerular basement membrane, so-called “hump”, covered by a glomerular epithelial cell with many endocytic vacuoles.

Renal tissue analysis

The kidney biopsies from patients A and B and one of the 6 controls, a patient with IgA nephropathy, were positive for *S. pyogenes*.

Checkerboard DNA-DNA hybridization showed that both renal biopsies contained *S. constellatus*.

S. constellatus was positive for the control patients PL 1723-98, PL 10496-98 and PL 12992-98 but negative for the patients PL 400-98 and PL 12732-98. The latter five control-patients were negative for *S. pyogenes*. Their renal tissue contained, according to light microscopy, mainly nephrosclerosis.

DISCUSSION

This study describes an outbreak of glomerulonephritis associated with the isolation of *S. pyogenes* in two kidney biopsies, but also with identical strains of *S. constellatus* in throat cultures, and in both cases in renal biopsies, from the patients and members of the families and other families in the neighbourhood (Fig. 2). *S. constellatus* was also present in 3/6 control kidney biopsies. *S. constellatus* belongs to the normal flora of the

mouth from which it may spread. In the index patient (A) GCS were isolated but subtyping was not performed.

S. pyogenes was positive also in a kidney biopsy of one control patient with chronic glomerulonephritis (i.e. IgA nephropathy). The remaining 5 specimens were negative for *S. pyogenes*.

S. constellatus has also been found in abscesses and in peritonsillitis and in rare cases in association with pharyngitis [9–11].

The index patient (A) had a serious dialysis-requiring condition and a second patient (B) had a notable renal failure with a serum creatinine of 200 µmol/l. One child had glomerulonephritis with a nephrotic syndrome and another had glomerulonephritis (C, D). *S. pyogenes* may have been the main cause of glomerulonephritis in both of the kidney-biopsied patients. Whether *S. constellatus* was a concomitant cause of glomerulonephritis cannot be determined at present. Other bacteria than group A streptococci have increasingly been recognized as causes of acute post-infectious glomerulonephritis [16, 17]. An association between *S. constellatus* and glomerulonephritis has so far not been reported. The possibility of an infection with GAS or large colony type GCS, preceding the glomerulonephritis in the index patient and the three patients colonized with *S. constellatus* must be considered (Figs 4 a-b) even if throat cultures at the onset of tonsillitis were negative. This possibility is corroborated by the finding of *S. pyogenes* in the renal tissue of two patients. This possibility might also be supported by the increased levels of AST and anti-DN:ase B in two patients suggesting a recent infection with GAS or possibly GCS [18–20]. *S. constellatus* is not, to our knowledge, known to produce streptolysin O or DN:ase B.

Glomerulonephritis associated with large colony type GCS (*S. zooepidemicus*) has been described but is uncommon [5,6]. The mechanism is not known but may be the same as that proposed for glomerulonephritis associated with GAS, i.e. nephritogenic proteins (endostreptosin) produced by the bacteria [21]. Both of our kidney biopsied patients had a picture of acute diffuse proliferative glomerulonephritis of the kind seen in acute poststreptococcal glomerulonephritis. In acute post-streptococcal glomerulonephritis, NSAP (nephritogenic associated protein) with production of streptokinase from the streptococci has been suggested to induce the binding of complement to the glomeruli by itself and by a specific antibody [22–24]. If *S. constellatus* caused the glomerulonephritis such a mechanism seems unlikely, since the *ska* gene was not present in the *S. constellatus* strains isolated here. The mechanisms of acute post-streptococcal glomerulonephritis are however still unclear [3, 13].

Recently a bacterial protein H, a surface protein of *S. pyogenes* has been discussed in the pathogenesis of acute poststreptococcal glomerulonephritis [25].

Circulating immune complexes with deposition and induction of complement and inflammatory cells in glomeruli as well as *in situ* antigen-antibody reactions are considered as the main mechanism of postinfectious glomerulonephritis, and bacterial antigens not present in GAS-induced nephritis may have been involved [3, 13].

S. constellatus is not known to induce tonsillitis, but may occur in peritonsillar abscesses and periapical endodontic lesions of asymptomatic teeth [26, 27]. It may also be present in empyema thoracis and lung abscess and has been described in association with endocarditis [28, 29]. Glomerulonephritis has been described also in association with endocarditis caused by α-hemolytic (viridans) streptococci [30–32]. Such streptococci are however more able to induce chemotaxis of leucocytes than the streptococci belonging to the milleri group in which *S. constellatus* is included [33]. The presence of *S. pyogenes* in the biopsy of the control patient with IgA nephropathy may be coincidental. A connection between IgA nephropathy and post-streptococcal glomerulonephritis in some cases is suggested in the literature [34, 35]. This may also be the case in Hennoch-Schönlein´s purpura, a disease related to IgA nephropathy [36]. The epidemic described suggests either that the outbreak of four cases of glomerulonephritis was due to *S. pyogenes* but coincided with the transmission and colonization of *S. constellatus* or that *S. constellatus* strains were highly pathogenic or nephritogenic and that this organism can be effectively transmitted. Further studies on the possible role of *S. constellatus* in the pathogenesis of post-streptococcal glomerulonephritis [37] are warranted.

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