# Hepatitis C quantification and sequencing in blood products, haemophiliacs, and drug users

PETER SIMMONDS LIN QI ZHANG HENRY G. WATSON
SELMA REBUS ELAINE D. FERGUSON PETER BALFE
GLYNIS H. LEADBETTER PENG LEE YAP JOHN F. PEUTHERER
CHRISTOPHER A. LUDLAM

**The polymerase chain reaction (PCR) detected specific hepatitis C viral (HCV) RNA sequences in plasma from 15 of 21 haemophiliacs (12 HCV-antibody positive) and 7 of 27 intravenous drug users (13 HCV-antibody positive). Quantification of RNA-positive samples showed high levels of HCV ($10^5$ to $10^6$ copies of RNA/ml) in infected patients. HCV was more frequently found in haemophiliacs infected with human immunodeficiency virus (11/11 HIV-positive and 4/10 HIV-negative patients). HCV-RNA was detected in all batches of commercially available factor VIII tested and in low concentrations in some pools of plasma donations from volunteers. Factor VIII, manufactured from volunteer donations, was uniformly negative by PCR. Phylogenetic analysis of viral sequences showed two distinct groups: one was associated with intravenous drug users and the other with haemophiliacs infected with Scottish factor VIII preparations. Both were distinct from sequences found in commercially available factor VIII.**

*Lancet* 1990; **336**: 1469–72.

## Introduction

Hepatitis C virus (HCV)[1] has been identified as an important cause of non-A, non-B (NANB) post-transfusional hepatitis.[2] Many epidemiological studies are based upon the Ortho enzyme immunoassay (EIA) but, there may be a delay of up to a year between exposure to HCV and seroconversion.[2] Furthermore, this test may give false-positive results.[3] An anti-HCV recombinant immunoblot assay (RIBA) failed to confirm the presence of specific antibody in over 70% of EIA-reactive blood donations.[4]

HCV-RNA sequences have been found in liver[5] and plasma[6] of infected individuals with the polymerase chain reaction (PCR). We now report on the relation between HCV viraemia (measured by PCR) and HCV antibody status, together with RNA quantification and sequencing, in haemophiliacs and intravenous drug users (IVDUs).

## Patients and methods

### *Patients*

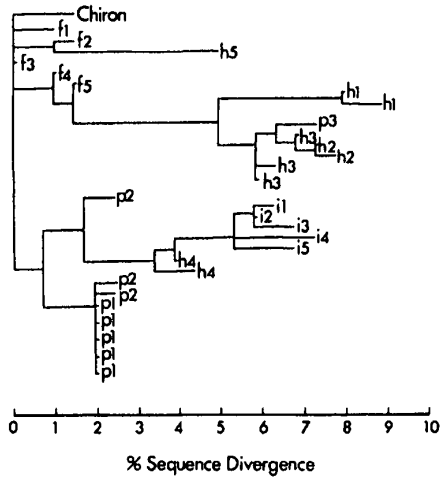
Plasma samples from 21 haemophiliacs were stored at −70°C before PCR analysis. Sera from 27 IVDUs were kept at 4°C for 3–7 days with long-term storage at −20°C before testing. Blood products were obtained from the National Institute of Biological Standards and Controls, and the Protein Fractionation Centre, Edinburgh. Freeze-dried preparations (factor VIII, factor IX) were stored at 4°C before reconstitution. Serum and plasma samples were tested by the Ortho EIA for HCV antibodies. Results are given as the optical density (OD) of the test sample divided by control OD to give the OD index (ODI). A sample was EIA-positive if ODI > 1. Most samples were further tested by the Ortho RIBA.

200 µl plasma or reconstituted blood products, 600 µl denaturing solution D, and 600 µl water-saturated phenol were mixed[7] and supplemented with 1 µg/ml purified carrier RNA (sheep fibroblast). 100 µl chloroform was added and the tube incubated on ice for 15 min. After centrifugation (14 000 *g*, 4°C, 15 min), the aqueous phase was removed and re-extracted with an equal volume of chloroform. RNA was precipitated with an equal volume of isopropanol at −20°C to −70°C for a minimum of 2 h. After further centrifugation (14 000 *g*, 4°C, 15 min), RNA was washed in 1 ml 70% ethanol solution, air-dried at 45°C, and redissolved in 20 µl water. RNA from larger samples (1 ml) was extracted by dilution with 9 ml phosphate-buffered saline, ultracentrifugation (50 000 *g*, 4°C, 3 h), and by the method described above.

### *RNA detection, quantification, and sequencing*

Sense (ED1, GTGGTCGACTGCAATACGTGTGTCAC) and antisense (ED2, CCGGCATGCATGTCATGATGTAT) primers were used for the first reaction in a double PCR. ED3 (CACCCAGACAGTCGATTTCAG) and ED4 (GTATTTGGTGACTGGGTGCGTC) were inner (nested) primers used for the second reaction. Some samples were amplified with primers d94, d95, N1, and N2.[6] However, these primers consistently detected fewer positive samples than ED1–ED4—eg, 4/13 IVDUS were positive with d94–d95, compared with 7/13 positives with primers ED1–ED4, and were not used further. cDNA synthesis of 3 µl RNA was carried out at 42°C for 30 min with 7 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, USA) in 50 mmol/l "tris"-HCl, pH 8·0; 5 mmol/l $MgCl_2$; 5 mmol/l dithiothreitol; 50 mmol/l KCl; 0·05 µg/µl bovine serum albumin (molecular biology grade, BCL, Lewes, UK); 600 µmol/l dATP, dCTP, dGTP, and TTP; 15% dimethyl sulphoxide; 1·5 µmol/l primer ED2; 0·1 µg/µl carrier RNA (sheep fibroblast); and 10 U RNAsin (Promega). Part of the cDNA (4 µl) was amplified over twenty-five cycles with primers ED1 and ED2 in 50 µl PCR buffer.[8] 1 µl of product was amplified for a further twenty-five cycles with ED3 and ED4. Amplified DNA was detected by agarose-gel-electrophoresis and ethidium bromide staining. Quantification was by limiting dilution analysis of cDNA reverse transcribed from RNA.[8] Sequence analysis was carried out as described previously.[9] Each sequence was read in both directions, by priming with either ED3 or ED4. Phylogenetic analysis by the maximum likelihood procedure was completed with the Phylip package.[10] Sequence differences were taken as significant if the inferred G statistic exceeded the tabulated value at a probability of

ADDRESSES **Department of Medical Microbiology, University of Edinburgh, Teviot Place, Edinburgh** (P Simmonds, BM, S. Rebus, BSc, G. H Leadbetter, FIMLS, J F Peutherer, FRCPath); **Department of Genetics, University of Edinburgh, Kings Buildings, Edinburgh** (L Q Zhang, MSc, P Balfe, PhD, E. D Ferguson, BSc); **Department of Haematology, Royal Infirmary, Lauriston Place, Edinburgh** (H G Watson, MRCP, C A Ludlam, FRCP); **and Edinburgh and South-East Scotland Regional Blood Transfusion Service, Lauriston Place, Edinburgh, UK** (P L Yap, MRCPath) Correspondence to Dr C A Ludlam, Department of Haematology, Royal Infirmary, Lauriston Place, Edinburgh EH3 9YW, UK




**Sequence diversity of HCV.**

Chiron, published sequence; f 1–5, 5 commercial factor VIII batches; h 1–5, 5 haemophiliacs; i 1–5, 5 Edinburgh IVDUs; p 1–3, 3 Scottish plasma pools

0·01. All distances indicated in the figure were significant by this method.

PCR sensitivity was assessed by equivalent experiments to those described above, but with cloned herpes simplex virus thymidine kinase (tk) gene[11] (unpublished data). An estimated 10% of cDNA transcripts extended sufficiently to be amplifiable by a pair of primers spaced 260 base-pairs apart. Equivalent experiments with known amounts of human immunodeficiency virus RNA yielded efficiencies of reverse transcription that varied with the spacing of the primers. By extrapolation we calculated an overall efficiency for detection of HCV-RNA sequences with ED1-ED4 of 5%.

## Results

### *HCV detection and quantification*

HCV antibody (EIA and RIBA) and RNA status in both IVDUs and haemophiliacs are shown in table I. HCV-RNA was more frequently detected in haemophiliacs who were also infected with human immunodeficiency virus type 1 (HIV-1). Of the 15 PCR-positive haemophiliacs, 11 were HIV-positive, whereas all 6 PCR-negative haemophiliacs were HIV seronegative. All 4 of the PCR-positive haemophiliacs who had no detectable antibody to HCV had HIV-related symptoms at the time of plasma collection, and had CD4 lymphocyte counts of $< 2 \times 10^5$/ml. The average CD4 count of the PCR-positive group was $250 \times 10^6$/l, and in the negative group $550 \times 10^9$/l ($p < 0{\cdot}05$). No association between HCV infection and either annual factor VIII use or increases in alanine aminotransferase (ALT) was found.

The amount of RNA in samples from 4 positive IVDUs and 4 haemophiliacs was estimated by titration of cDNA. PCR with nested primers detects single molecules of target DNA sequence. Tests on multiple replicates at a suitable limiting dilution give a Poisson distribution of positive and negative results that reflects the concentration of target DNA.[8] To obtain an RNA concentration from quantification of cDNA, we have assumed an overall efficiency of 5% for the reverse transcription step. The amounts of circulating RNA in 4 haemophiliacs and 4 IVDUs ranged from $3{\cdot}5 \times 10^4$ to $1{\cdot}1 \times 10^6$ copies of RNA/ml. The amounts recovered in IVDUs were similar to those of haemophiliacs. The cutoff limit of this assay was $4 \times 10^3$ copies of RNA/ml.

TABLE I--ANTIBODY (EIA AND RIBA) AND HCV-RNA STATUS IN IVDUs AND HAEMOPHILIACS

| | IVDUs (n = 27) | | Haemophiliacs (n = 21) | |
|---|---|---|---|---|
| -- | Ab + | Ab − | Ab + | Ab − |
| PCR RNA + | 7 | 0 | 11 | 4* |
| PCR RNA − | 6 | 14 | 1 | 5† |

*1 sample EIA-positive (ODI > 5·8) but RIBA-negative
†1 sample EIA-positive (ODI = 1·04) but RIBA-negative

TABLE II—HCV-RNA STATUS IN BLOOD PRODUCTS

| | Factor VIII | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| PCR RNA | Commercial* | Scottish BTS† | UK non-commercial‡ | Commercial heat-treated§ | Scottish BTS heat-treated¶ | Factor IX† | IV IgG† | Plasma pools** |
| + | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| − | 0 | 4 | 5 | 1 | 4 | 5 | 6 | 4 |

BTS = blood transfusion service, *expiry date 1980–83, †volunteer donations 1983, ‡volunteer donations 1982–83, §wet heat-treated (60°C, 20h), ¶dry heat-treated (80°C, 72h), and **1000 Scottish blood donations 1990

All unheat-treated commercial batches factor VIII tested were RNA-positive (table II). Quantification by limiting dilution of two of these samples gave RNA concentrations in the original material of $2 \times 10^4$ to $10^5$ copies of RNA/ml. All 9 of the non-commercial factor VIII concentrates prepared from volunteer blood donations were negative. The cutoff limit of the PCR assay for initial screening was 2000 RNA molecules/ml. To investigate whether factor VIII prepared from volunteer blood contained quantities of HCV below the threshold of sensitivity for the RNA PCR, RNA from larger volumes of Scottish concentrate was retested in an assay with a cutoff sensitivity of 200 copies of RNA/ml. All 4 batches tested remained negative. All batches of factor IX and intravenous IgG were negative for HCV-RNA. To examine whether these negative results were due to an absence of infectious blood donors, seven plasma pools that each contained 1000 volunteer donations, were tested by the PCR. Three of seven pools were positive for HCV-RNA. Samples contained 200–1900 copies of RNA/ml (test threshold 200 copies/ml). This result suggested an average frequency of < 1 PCR-positive donation/thousand in the local donor population. All plasma pools were negative for HCV antibody by the Ortho EIA (ODIs < 0·2). However, as antibody titres in this test were extremely low in positive sera (ranging from only 1/10 to 1/100 in sera from 8 IVDUs) the Ortho EIA cannot detect contamination of plasma pools by low numbers (< 10) of antibody-positive donations.

### *Phylogenetic analysis*

Nucleotide sequencing of HCV was completed by isolation of single cDNA molecules, amplification with nested primers ED1–ED4, and direct sequencing.[9] Sequences corresponded to those for a non-structural protein homologous to NS3 in flaviviruses.[12] Nucleotide substitutions were seen at 42 of 216 sites. No gaps or stop codons that would interrupt the reading frame of the nucleotide sequence were found. Almost all nucleotide changes left the encoded peptide sequences unchanged (synonymous substitutions). 17 times more synonymous nucleotide substitutions were in HCV sequences than would be expected in randomly mutating DNA,[13] which indicates a strong selection pressure on HCV against

changes in the encoded protein. Almost all substitutions were transitions, whereby a purine is substituted for another purine, or a pyrimidine is substituted by a pyrimidine.

The likely evolutionary relations between the variants of HCV are shown in the figure. The extent of differences (evolutionary distance) between variants is shown by horizontal lines. Closely related sequences are found in all IVDUs (i1-5), which suggests that they were infected from a common source. These sequences are also similar to two from an 8-year-old haemophiliac, who was first infected from factor VIII produced in Scotland in 1984 or 1985 (h4). Three haemophiliac sequences (h1–3), and an HCV sequence present in one of three positive plasma pools (p3) were also closely related, but distinct from the IVDU group. These 3 haemophiliacs may have been infected at the same time from cryoprecipitate in the mid-1960s. Sequences in two other plasma pools (p1, p2) form a third closely related group. All five batches of commercial factor VIII (f1–5) contained similar sequencies to that of the published HCV genome.[14] Finally, one sequence from an Edinburgh haemophiliac (h5) was highly divergent from the commercial factor VIII group, and from the 2–3 groups identified in Edinburgh individuals.

## Discussion

Haemophiliacs had higher rates of detectable HCV infection by PCR (15/21) than by antibody tests (12/21). Of the 5 who showed no evidence of infection, 1 was a 3-year-old boy who received dry heat-treated concentrate only. The other 4 were moderate to severe haemophiliacs with high annual factor VIII use (>19 000 U/year; mean 3 years); all previously received unheated concentrates and have intermittently or persistently increased ALTs. The observation that 1 of the 4 seronegative haemophiliacs was previously antibody positive (ODI 3·2 in 1987 and 2·2 in 1988) suggests that these individuals had been infected with HCV, but had cleared the virus more rapidly than the others after the introduction of heat-treated factor VIII in 1985. The continued liver enzyme abnormalities may be due to an uncharacterised viral agent. Alternatively, as titres of antibody in the Ortho EIA are extremely low in positive samples (1/10–1/100), apparent sero-reversion may take place despite continued infection. Weiner et al[5] failed to detect HCV antibody in 2 patients with chronic posttransfusional liver disease, despite finding HCV-RNA in liver tissue.

Higher rates of HCV infection were found in haemophiliacs infected with HIV. Haemophiliacs who were PCR-positive and antibody-negative all had AIDS and low CD4 cell counts ($<200 \times 10^9$/l). Negative antibody results may be due to either this immunodeficient state or to high rates of viral protein expression that could adsorb circulating antibody by immune complex formation.

In contrast to the haemophiliacs, almost half of IVDU antibody-positive sera were negative by PCR. The use of serum rather than plasma, and the uncertain storage conditions of IVDU samples may have contributed to the failure to detect HCV-RNA. Alternatively, the viraemia may have been below the level of detection of the assay because the lower limit of the observed range was close to the assay threshold.

The finding of high rates of HCV-RNA in infected individuals is not consistent with a previous report that only 1 of 6 HCV antibody-positive blood donations transmitted infection to recipients.[6] It is possible that only individuals with high levels of circulating HCV are infectious but this is not consistent with the high rates of HCV infection associated with use of English and Scottish factor VIII that have low or undetectable levels of HCV-RNA[15] (table II). It is more likely that the 5 non-infectious, HCV-RNA donations had given false-positive results in the Ortho EIA.

The considerable sequence diversity of HCV in different patient groups resembled that of other RNA viruses with geographically separated variants—eg, the *pol* gene sequences of African and North American HIV-1.[16,17] HCV sequences were divided into three distinct groups: IVDUs (il-5), locally infected haemophiliacs (h1–3), and those who received commercial factor VIII concentrates (f1–5). The finding of related sequences in IVDUs is not surprising because needle sharing was common. The close relation between the second group of sequences obtained from five batches of factor VIII from different manufacturers is surprising in view of the wide geographical area from which paid donations are collected.

3 of 5 Edinburgh haemophiliacs were infected with a different HCV variant which was also found in one of the Scottish blood donations (p3). These 3 individuals are aged 20–30 years and were probably first infected in the 1960s with locally collected fresh frozen plasma or cryoprecipitate. These 3 individuals differ from h4, who is now aged 8 and was infected no earlier than 1984 or 1985, and from h5, who has received commercial factor VIII. The group h1–3 may consist of a variant of HCV common in Scotland 25 years ago with the IVDU/h4 sequence types becoming more prevalent in the early 1980s. However, it is not known whether reinfection with HCV can take place, and it is possible that variants in h1–3 are the results of reinfection at any time between the mid-1960s and mid-1980s.

We thank Dr G. Kemball-Cook and Dr D. P. Thomas for samples of commercial and UK non-commercial factor VIII for PCR testing; Dr R. J. Perry, Dr H. Hart, and Dr A. Todd for the supply of blood products manufactured by the Protein Fractionation Centre, Edinburgh; and Dr R. Al-Shawi for cloned tk sequences and tk primers.

### REFERENCES

1. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359–62.
2. Alter HJ, Purcell RH, Shih JW, et al. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N Engl J Med* 1989; **321**: 1494–500.
3. MacFarlane IG, Smith HM, Johnson PJ, Bray GP, Vergani D, Williams R. Hepatitis C virus antibodies in chronic active hepatitis: pathogenic factor or false-positive result? *Lancet* 1990; **335**: 754–57.
4. Skidmore S. Recombinant immunoblot assay for hepatitis C antibody. *Lancet* 1990; **335**: 1346.
5. Weiner AJ, Kuo G, Bradley DW, et al. Detection of hepatitis C viral sequences in non-A, non-B hepatitis. *Lancet* 1990; **335**: 1–3.
6. Garson JA, Tedder RS, Briggs M, et al. Detection of hepatitis C viral sequences in blood donations by "nested" polymerase chain reaction and prediction of infectivity. *Lancet* 1990; **335**: 1419–22.
7. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–59.
8. Simmonds P, Balfe P, Peutherer JF, et al. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. *J Virol* 1990; **64**: 864–72.
9. Simmonds P, Balfe P, Ludlam CA, Bishop JO, Leigh Brown AJ. Analysis of sequence diversity in hypervariable regions of the external glycoprotein of human immunodeficiency virus type 1. *J Virol* 1990; **64**: 5840–50.
10. Felsenstein J. Phylogenies from molecular sequences: inference and reliability. *Ann Rev Genet* 1988; **22**: 521–65.
11. Al-Shawi R, Burke J, Jones CT, et al. A *Mup* promoter-thymidine kinase reporter gene shows relaxed tissue-specific expression and confers male sterility upon transgenic mice. *Mol Cell Biol* 1988; **8**: 4821–28.

12. Miller RH, Purcell RH. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. *Proc Natl Acad Sci USA* 1990; 87: 2057–61.
13. Li W-H, Wu C-I, Luo C-C. A new method for estimating synonymous and nonsynonymous rates of nucleotide substitution considering the relative likelihood of nucleotide and codon changes. *Mol Biol Evol* 1985; 2: 150–74.
14. Houghton M, Choo QL, Kuo G. *Eur Patent Appl* 88, 310, 992.5 and *Publ* 318, 216.
15. Garson JA, Preston FE, Makris M, et al. Detection by PCR of hepatitis C virus in factor VIII concentrates. *Lancet* 1990; 335: 1473.
16. Alizon M, Wain-Hobson S, Montagnier L, Sonigo P. Genetic variability of the AIDS virus: nucleotide sequence analysis of two isolates from African patients. *Cell* 1986; 46: 63–74.
17. Starcich BR, Hahn BH, Shaw GM, et al. Identification and characterisation of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. *Cell* 1986; 46: 637–48.

# Effacement of glomerular foot processes in kwashiorkor

MICHAEL H. N. GOLDEN STANLEY E. H. BROOKS DAN D. RAMDATH
ELAINE TAYLOR

**In a study of the pathogenesis of the oedema of kwashiorkor the ultrastructure of the kidneys from 6 children was examined shortly after they died from oedematous malnutrition. There was a generalised effacement of the glomerular epithelial cells onto the basement membrane. The filtration slits that remained were narrowed. The picture was similar to that seen in minimal-change nephrotic syndrome—but none of the children had albuminuria. The degree of effacement was statistically related to treatment with gentamicin. The findings suggest that there is a defect in the anionic charge of the glomerular basement membrane in oedematous malnutrition, that the membrane charge is more easily neutralised by cations such as gentamicin, and that, because proteinuria is not a feature of oedematous malnutrition, the proteinuria in other conditions associated with glomerular epithelial cell effacement (eg, minimal-change nephrotic syndrome) is due to something more complex than simple loss of charge.**

*Lancet* 1990; **336**: 1472–74.

## Introduction

The kwashiorkor syndrome is a generalised disorder characterised by oedema. There are changes in the cellular metabolism of sodium and potassium[1] and abnormalities in the renal handling of salt, water, acid, and osmolal loads; proteinuria, however, is uncommon.[2,3] How these abnormalities arise is unknown.[4] We have thus examined the ultrastructure of kidneys taken immediately after death from children with oedematous malnutrition.

## Subjects and methods

6 children aged 4–18 months, were studied. 3 had kwashiorkor and 3 marasmic-kwashiorkor; all had nutritional oedema, fatty liver, and bacterial infection, and all had been treated according to published regimens.[5] Their plasma urea (range 1·0 to 2·8 mmol/l) and plasma creatinine (30 to 100 μmol/l) were not raised, and no patient had proteinuria. The only recognised nephrotoxin given to the children was gentamicin (5 mg/kg per day). Tissues taken within 2 h of death (table) were fixed in glutaraldehyde and processed by standard procedures. Sections from at least three blocks were stained with uranyl acetate and lead citrate.[6] Between 15 and 19 positive prints were recorded of glomeruli from each child. These prints were coded and scored blindly. A score of 0 to 4 was assigned to each photograph on the basis of whether there were no filtration slits (0), occasional narrow slits (1), moderate narrow and/or occasional normal slits (2), numerous narrow or moderate normal slits (3), or normal filtration slits (4). The scores for each picture from a subject were added up and expressed as a percentage of the maximum score achievable: a low score thus represents podocyte foot-process effacement.

The study was approved by the ethnics committee of the University of the West Indies.

## Results

Case 5 had terminal acute renal failure secondary to hypovolaemia. None of the other children showed clinical evidence of renal abnormality.

### *Light microscopy*

Light microscopy did not reveal any abnormality of the glomeruli or tubular necrosis except in case 5, in which there was some suggestion of mesangial proliferation, and in which the proximal tubules showed oedema and inclusion bodies with tubulorrhexis.

### *Electron microscopy*

All sections showed glomerular basement membranes of uniform thickness, with clear delineation of the three laminae. No dense deposits were present in the glomerular loops or in the mesangium (fig 1). Both visceral and parietal epithelial cells were prominent. There were varying degrees of epithelial cell oedema, but vacuolation was not a feature. In all subjects the epithelial cell foot processes were effaced, (fig 1) and occurred throughout the glomeruli (fig 2). The degree of effacement varied between patients (table) but within an individual the pattern of effacement was uniform. Many of the filtration slits were narrow.

Simple regression analyses were done with height, weight, age, gentamicin total dose (per kg), time-on-gentamicin, length of time from stopping gentamicin to death, time from admission to death, admission urea, and admission creatinine as the independent variables and the podocyte effacement score as the dependent variable. Multiple regression analysis was then done with the variables most closely associated with the podocyte score. None of the variables except those associated with

ADDRESSES **Tropical Metabolism Research Unit** (M H N Golden, FRCP, D D. Ramdath, PhD), **and Department of Pathology** (S E H Brooks, FRCPath, E. Taylor, DMT), **University of the West Indies, Mona, Kingston 7, Jamaica, West Indies.** Correspondence to Dr M H N Golden.