Background. Chronic kidney disease (CKD) patients show evidence of chronic inflammation with mononuclear cell activation which is mainly caused by uraemia itself and is exacerbated by haemodialysis. Small fragments of bacterial DNA (DNAb) are ubiquitous contaminants, which are capable of passing through dialyser membranes causing the stimulation of cells of the immune system. The aim of this study was to evaluate whether DNAb contamination may have an effect on apoptosis of activated monocytes from CKD-5 patients.

Methods. To test the ability of DNAb to stimulate the inflammatory response, mononuclear cells from 10 chronic kidney disease patients who had not begun haemodialysis (ND-CKD-5) and 10 patients undergoing regular dialysis (HD) were cultured in the presence and absence of DNAb. Ten healthy subjects were used as controls.

Results. The percentage of IL-1β cells was higher in HD patients than in ND-CKD-5 (33.9 ± 3.0% vs 20.0 ± 2.3%, P < 0.001) and controls (9.4 ± 2.1%, P < 0.001). The presence of DNAb induced an increase in the percent of cells expressing IL-1β in controls, ND-CKD5 and HD patients. In addition, the DNAb also increased the release of cytokines in all groups, the effect was more marked in ND-CKD5 and HD than in controls. DNAb only inhibited apoptosis of activated mononuclear cells from, ND-CKD5 and HD patients (27 ± 2.5% vs 14.6 ± 2.9%, P < 0.01).

Conclusions. DNAb enhances cytokine production and promotes the survival of inflammatory mononuclear cells from CKD patients. These results strongly suggest that DNAb fragments play an important role in maintaining chronic inflammation in patients on haemodialysis.
detected in dialysate [8,9]. Therefore, a relevant question for the clinician is whether the presence of small amounts of DNAb will cause any significant biological response in the patient [10]. DNAb has been shown to inhibit apoptosis of dendritic and B cells [11,12]; DNAb could also inhibit apoptosis of activated monocytes. Thus, even small amounts of DNA fragments may potentially contribute to the prolonged life span of inflammatory cells in CKD patients on regular haemodialysis [13,14].

The aim of the present study is to evaluate the effect of bacterial DNA on both apoptosis and inflammatory activity of mononuclear cells from CKD patients.

**Subjects and methods**

**Determination of bacterial DNA in the dialysate**

The water used in our dialysis unit was considered sterile: levels of endotoxins in the dialysis fluids were always lower than 0.03 UE/ml and the bacterial contamination was lower than 0.1 CFU/ml (colony-forming unit/millilitre).

Dialysis fluids were analysed for fragments of Gram-negative bacteria DNA by electrophoresis of gel agarose. Semiquantitative PCR (polymerase chain reaction) was used to detect the gene coding for the bacterial 16S-rRNA in different solutions. Primers were designed to amplify the 16S-rRNA gene common for all Gram-negative bacteria. Amplified PCR products were analysed by electrophoresis in 1% agarose gel and stained with ethidium bromide. DNAb was determined in 50 ml of dialysis fluid at three different sites: (i) at the connection port of the distribution ring just before water enters the dialysis monitor; (ii) at the dialyser inlet and (iii) at the dialyser outlet. These measurements were performed in triplicate on four different days. Three millilitres of a solution of bacterial DNA, at a concentration of 1.8 mg/ml, was used as positive control.

**Subjects**

Two groups of patients were studied: the first group included 10 consecutive CKD-5 patients (ND-CKD-5) before the initiation of regular haemodialysis therapy; the second group was comprised of 10 haemodialysis patients (HD) chosen at random from the Dialysis Unit of Reina Sofia University Hospital (Cordoba, Spain). In both groups, the exclusion criteria were as follows: patients older than 70, diabetics, those with PTH > 400 pg/ml, evidence of infection, neoplasia, immune or liver disease. Patients on vitamin D, non-steroidal anti-inflammatory drugs (NSAIDs), angiotensin-converting enzyme inhibitors (ACEI), angiotensin II receptor antagonists (ARAII) or immunosuppressive drugs were also excluded. Ten healthy subjects served as controls. All patients were informed of the objectives of the study and all of them consented to participate.

The ND-CDK-5 group consisted of 10 subjects (three females and seven men) with a mean age of 46.5 ± 12.3 years (24–63), diagnosed with CKD who had not started dialysis, with a creatinine clearance ranging from 5 to 15.3 ml/min (9.45 ± 3.3) calculated according to the MDRD4 formula.

Ten patients (four females and six men) with a mean age of 43 ± 13.0 years (25–69) on HD treatment using a 1.8 m² haemophan membrane (GFS 20 Gambro®, Hechingen, Germany) were included. All patients were dialysed with this dialyser for at least 2 months before being included in the study. Mean dialysis time on maintenance HD was 42.0 ± 19 months. The dialysis dose expressed as eKt/V was 1.48 ± 0.15, the reduction urea ratio was 74.12 ± 5.33% and the protein catabolic rate was 1.25 ± 0.37 g/kg/day. All patients were dialysed through a native arteriovenous fistula. In all cases, the dose of erythropoietin was adjusted to maintain a predialysis haemoglobin level higher than 11.5 g/dl.

There were no statistically significant differences in age and gender among the three groups studied (Table 1).

**Isolation of mononuclear cells from peripheral blood**

A sample of 10 ml of peripheral venous blood was drawn under sterile conditions from patients and healthy subjects. Blood samples were placed into tubes containing ethylene diamine tetra-acetic acid (EDTA). In the case of HD patients, the sample was always obtained from the arteriovenous fistula immediately before the first weekly dialysis. Peripheral blood mononuclear cells (PBMCs) were then isolated by density gradient separation on Ficoll (Ficoll/Hypaque, BioWhittaker Inc. Walkersville, MD, USA).

**Cell culture**

Mononuclear cells were cultured at 37°C in an atmosphere of 6% CO₂ in RPMI 1640 (BioWhittaker Inc. Walkersville, MD, USA), supplemented with glutamine (2 mM), sodium pyruvate (1 mM), streptomycin (50 g/ml), penicillin (100 IU/ml) and 10% fetal bovine serum. The fetal bovine serum was heated to 56°C for 60 min in order to inactivate the complement. Samples of 2 × 10⁵ mononuclear cells per well were placed on culture wells. Cells were cultured with and without DNAb. The DNAb fragments were sonicated (DNA type III of E. coli strain B Lot 119H4073, Sigma-Aldrich Quimica, S.A. Madrid, Spain) and added at a concentration of 1.8 µg/ml. Cells, were maintained in culture for a 48 h period.

**Intracellular cytokines**

Intracellular cytokine production was measured after 48 h of culture. For these studies, 600 µl blood samples were

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<th>Table 1. Demographic characteristics of the patients</th>
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Values are given as means±SD and range. ND CKD-5, non-dialysis CKD-5 patients who had not commenced dialysis; HD, haemodialysis patients.
incubated at 37°C in 15 ml polypropylene tubes (Nunc A/S, Denmark) in the presence of brefeldin A at 2 μg/ml (Sigma, St Louis, MO, USA). After incubation, cells were washed, fixed and permeabilized using FACS™ Permeabilizing Solution (Cat. No. 340973, Becton Dickinson Bioscience, San José, CA, USA). Thereafter, cells were incubated with 5 μl phycoerythrin (PE) conjugated antibody anti-IL-1β (Anti-Hu-IL-1βPE, code MG101, Caltag Lab., CA, USA) or the corresponding isotype control for 30 min in darkness at ambient temperature. Following incubation, the cells were washed and resuspended in 0.5 ml of Cell Fix™ (Cat. no. 340181, Becton Dickinson Bioscience). Cytofluorometric analysis was performed with a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Determination of IL-1β in supernatant and serum

IL-1β in supernatant and serum was determined by cytometry. The cytometric bead array technique is based on the binding between microparticles labelled with different fluorescence intensities and antibodies. The maximum wavelength emission was about 650 nm (fluorescence-3 channel, FL-3). The particles were bound by a covalent bond to an antibody against one of the cytokine IL-1β cytokines (Pharmingen, San Diego, CA, USA). The cytokines were captured directly in the immunoassay using different antibodies bound to PE, which emitted at 585 nm (fluorescence-2 channel, FL-2). The PE-conjugated detector antibody completed the sandwich. The intensity measured in (fluorescence-2 channel, FL-2). The PE-conjugated detector antibody completed the sandwich. The intensity measured in FL-2 was proportional to the concentration of cytokines in the sample, which was quantified from a calibration curve.

Cell apoptosis

Mononuclear cell apoptosis was measured using a kit that was based on the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) (Boehringer Mannheim). In accordance with the manufacturer’s instructions, 10⁶ cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed and permeabilized for 2 min in ice with 0.1% Triton X-100. After washing, cells were decanted and resuspended in 50 μl of TUNEL reaction mixture (5 μl of TUNEL enzyme-containing TdT mixed with 45 μl of TUNEL label-containing PE-dUTP and dNTP nucleotides) or in 50 μl of TUNEL Label, which served as the negative control. After 60 min at 37°C in a humid atmosphere, cells were washed three times in wash buffer (PBS + 0.1% NaN₃ + 10% autologous serum) and analysed by FACScalibur flow cytometer.

Statistics

All the data were expressed as means ± SD. Non-parametric data were compared by Kruskal–Wallis test. The Mann–Whitney U-test for unpaired data and the Wilcoxon signed rank test for paired data were used to compare means. Chi-square was used for qualitative variables. Values were regarded as significantly different at the P < 0.05 level. The statistical analyses were performed using the SPSS statistical package, version 9.1.

Results

Presence of bacterial DNA in the dialysis fluid

DNAβ was detected in all samples of the dialysis fluids examined including the dialyser inlet and outlet. A representative agarose gel electrophoresis of four DNAβ studies is shown in Figure 1. DNAβ fragments were not found in RPMI medium which was used as control.

Pro-inflammatory cytokines in CKD-5 patients

As compared with healthy controls, the non-dialysis CKD-5 (ND-CKD-5) patients had elevated serum levels of IL-1β (1208 ± 68 vs 510 ± 69 pg/ml, p < 0.009) (Figure 2). In haemodialysis patients, IL-1β values were even higher (2478 ± 205 pg/ml; P = 0.016) than in ND-CKD-5.

Effects of bacterial DNA on inflammatory activity

In both ND-CKD-5 and HD patients the percent of cells expressing intracellular IL-1β was greater than in healthy controls (20 ± 2.3% and 33.9 ± 3.0% vs 9.4 ± 2.1%; P < 0.001) (Figure 3). The percent of cells expressing intracellular IL-1β was greater in HD than ND-CKD-5 patients (P < 0.001) (Figure 3). In controls, the addition of DNAβ produced an increase in the percent of cells expressing IL-1β. (from 9.4 ± 2.1% to 15.4 ± 2.2%; P = 0.005) (Figure 3).

Fig. 1. Representative agarose gel electrophoresis of DNAβ. DNAβ was detected at the three sites analysed: 1 = DI: at the connection port of the distribution ring just before water enters the dialysis monitor; 2 = I: at the dialyser inlet; 3 = O: at the dialyser outlet. No DNAβ fragments were found in RPMI medium used as control test.
Similarly, DNAb also increases the percent of cells expressing IL-1β in ND-CKD-5 (from 20±2.3 to 25.4±4.7; \( P = 0.016 \)) and in HD patients (from 33.9±2.3% to 37.0±3.2%; \( P < 0.05 \)). Furthermore, the percentage of cells expressing intracellular IL-1β after DNAb exposure was greater in HD patients than in the other two groups (Figure 3).

The mean level of IL-1β in the supernatant of cell cultures from HD patients (1722±153 pg/ml) was higher than that in the ND-CKD-5 patients (898±79 pg/ml) (\( P < 0.01 \)) and healthy controls (520±65 pg/ml) (\( P < 0.01 \)) (Figure 4). The addition of DNAb to the cell culture increased the release of cytokines in the three groups (\( P < 0.05 \)) (Figure 4).

**Effects of DNAb on cell apoptosis**

The effect of DNAb on mononuclear cell apoptosis is shown in Figure 5. In the absence of DNAb, the percent of cell apoptosis was significantly higher in the HD group (27±2.5%) than ND-CKD-5 (17.5±2.8%; \( P < 0.01 \)) and healthy controls (6.2±1.8%; \( P < 0.01 \)). The percentage of apoptotic cells from ND-CKD-5 patients was greater than in the control subjects (\( P < 0.05 \)). The addition of DNAb induced a decrease in mononuclear cell apoptosis in ND-CKD-5 (from 17.5±2.8% to 12.3±2.6%; \( P < 0.01 \)) and in HD patients (from 27±2.5% to 14.6±2.9; \( P < 0.01 \)). Monocytes from HD and ND-CKD in culture survived longer with than without DNAb added to the medium (data not shown). In healthy subjects, the addition of DNAb did not significantly affect
mononuclear cell apoptosis (6.2 ± 1.8% vs 7.9 ± 3.6%).
Representative flow cytometry histograms of mononuclear cells apoptosis are presented in Figure 6.

Discussion

Chronic inflammatory activity in CKD patients has been postulated as an important pathogenic factor in the development of cardiovascular disease, the main cause of morbidity and mortality in CKD patients [3]. In this study, we show that DNAβ fragments were present in theoretically sterile dialysis fluid and that DNAβ fragments may contribute to the prolonged life span of inflammatory cells from CKD patients on regular haemodialysis.

The precise mechanism of DNAβ action is not completely characterized. Bacterial DNA contains unmethylated cytosine–guanine dinucleotide, with two purine 5’ and two pyrimidine 3’ (CpG) terminations. These are structures required to interact with TLR-9 (DNAβ receptor) which triggers a cell signalling pathway including activation of the mitogen-activated protein kinases (MAPKs) and the nuclear factor-κB (NF-κB). Thus, DNAβ is not incorporated into the cell DNA but modifies protein synthesis through NF-κB activation [15]. In cultures of mononuclear cells from CKD-5 patients, the addition of DNAβ increased the inflammatory response, prolonging their life span and delaying their death by apoptosis. Therefore, we propose that the presence of bacterial DNA may cause both intensification and prolongation of the inflammatory response in CKD patients.

Preliminary results from a different population of patients revealed that 80% of haemodialysis patients have trace amounts of DNA in serum (data not show). In agreement with previous reports [16,17], we have observed that mononuclear cells from CKD patients are activated and these cells produce and release pro-inflammatory cytokines. One of the factors that may contribute to mononuclear cell activation in CKD patients on haemodialysis is the presence of contaminant agents in the dialysate [10,18]. In order to prevent contamination, dialysis fluids are subjected to strict sterilization procedures. However, using polymerase chain reaction we have detected the presence of bacterial DNA in ultrapure water. These results are not surprising since previous reports by others have demonstrated contamination of the dialysate by short bacterial DNA fragments [9]. Furthermore, Schindler et al. [10] reported that bacterial DNA might induce the release of cytokine by immunocompetent cells. In this study, we also show that activated mononuclear
cells from CKD patients further increase cytokine production after stimulation with DNA.

In the present study, we have shown that in vitro, bacterial DNA delayed apoptotic death in mononuclear cells from CKD patients. By doing so the survival of these cells is prolonged. The inhibition of mononuclear cell apoptosis has been proposed as a mechanism to maintain activated immune cells in the host with immune response [19]. Thus, bacterial DNA does not only inhibit apoptosis of neutrophils and lymphocytes [15–20,12] but also inhibits the apoptosis of activated mononuclear cells. Other bacterial products such as LPS have been demonstrated that can prolong the life span of monocytes by suppressing apoptosis [5]. The mechanisms by which bacterial DNA decreases the apoptosis of mononuclear cells from CKD patients are currently under investigation. It is possible that, as reported in granulocytes, bacterial DNA may have a direct effect on intracellular signals that mediate the inhibition of apoptosis in monocytes. Alternatively, bacterial DNA might act indirectly enhancing mononuclear cell survival via induction of inflammatory cytokines and/or maintaining immunocompetent cell viability at the site of inflammation [8].

Our results show that bacterial DNA promotes the survival of inflammatory mononuclear cells in CKD patients by suppressing apoptosis. It is interesting to stress that apoptosis is the physiological mechanism by which senescent cells are removed from the circulation, thereby preventing the survival of unwanted cells. Thus, bacterial products or cytokines may inhibit apoptosis of inflammatory cells to prolong the life span of monocytes available to respond against injury. However, this physiological mechanism may be undesirable as in the case of the chronic inflammation status of CKD patients. In conclusion, the results obtained in this study strongly suggest that the anti-apoptotic action of bacterial DNA in mononuclear cells from CKD patients may contribute to perpetuate inflammation in these patients.

References


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Conflict of interest statement. None declared.

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