Functional and immunological studies in children with chronic renal failure: the effects of uremia and dialysis treatment
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Chapter 10

Children with chronic renal failure have reduced numbers of memory B-cells.

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ABSTRACT

Reduced serum IgG and subclass levels have been demonstrated in children with chronic renal failure. To study possible causes of this reduction, we analyzed B-cell subset composition, T-helper cell frequencies and immunoglobulin (Ig) production capacity in vitro in children with chronic renal failure, with or without dialysis treatment.

Eight children treated with peritoneal dialysis (PD), 8 on hemodialysis (HD), 9 children not yet dialyzed (CRF) and 9 healthy children (HC) were studied. B-cell subsets were characterized by determining CD27, IgM, IgD and CD5 expression within the CD19⁺ population. Intracellular expression of IFN-γ, IL-2 and IL-4 in PMA/ionomycin-stimulated peripheral blood mononuclear cells (PBMC) was used to evaluate T-helper frequencies. The capacity of B-cells to secrete Ig in vitro was determined by measuring IgG₁, IgG₂ and IgM in culture supernatants of anti-CD2/CD28 moAb- or SAC/IL-2- stimulated PBMC.

Memory B-cell numbers (identified as CD19⁺ IgM⁺ IgD⁻ or CD19⁺CD27⁺ lymphocytes) were lower in HD compared to HC (p<0.05). CD19⁺ CD27⁺ B-cells were also significantly reduced in the PD population. Compared with HC, CD5⁺ (naive) B-cells were reduced in HD-treated patients but not for PD nor CRF children. No significant differences in CD4⁺ T-helper cell subsets were found between the groups. However, CRF children had a higher percentage IFN-γ producing CD8⁺ T-cell lymphocytes compared to HC (p=0.02). Finally, IgG₁, IgG₂ and IgM production in vitro was similar in the four groups.

In conclusion, significantly lower numbers of memory type B-cells were found in HD and PD children compared to healthy controls. This reduction may contribute to the low Ig levels found in these children.
INTRODUCTION

Reduced serum IgG and subclass levels have been described in children treated with peritoneal dialysis [1-4]. Peritoneal loss, reduced synthesis or increased catabolism, are possible explanations for this. In previous studies we have demonstrated that peritoneal loss is not the only explanation since reduced serum IgG and subclasses levels were already present before the dialysis treatment had started [5]. Most IgG is produced by specialized B-cells or plasma cells that have undergone class switching in the germinal center in response to T-cell dependent antigens. During this response, B-cells become activated by specific antigens, but depend on T-helper cells derived signals to differentiate into plasma cells and memory cells [6]. It is not clear whether the lower Ig levels in uremic patients are caused by intrinsic B-cell defects, disturbed helper T-cell activity, or a combination of both. B-cells express several surface molecules such as IgM, IgD, IgG, CD27, and CD5 that allow separation of naive (IgD+ or CD5+) and memory (IgD- or CD27+) type B-cells [7-11]. However, no information is available on B-cell differentiation in adult and pediatric patients with chronic renal failure, either or not treated with dialysis.

The T-helper cells derived cytokines, interferon-γ (IFN-γ), interleukin-2 (IL-2) and interleukin-4 (IL-4) are involved in B-cell maturation, Ig secretion and IgG subclass switching [12;13]. T-helper type 1 cells (Th1) produce mainly IFN-γ and IL-2. IL-4 is particularly produced by T-helper type 2 (Th2) cells [14]. The Th1/Th2 balance is crucial for an effective immune system and disturbances in the Th1/Th2 balance have been associated with diseases [14]. CD8+ (cytotoxic) T-cells also produce IFN-γ, IL-2 and IL-4 [14]. Data on cytokine production in uremia are controversial [15,16]. Most studies on cytokines in HD patients have focussed on the effect of hemodialysis modalities and of the type of hemofilter on cytokine production and Th1/Th2 balance [17,18]. In PD patients, cytokine production has been studied mainly in peritoneal dialysis effluent in relation to peritonitis episodes, the toxicity of the dialysis solution and peritoneal permeability [19-21]. To further explore possible causes of low IgG levels in uremic children, we measured B-lymphocyte immunoglobulin producing capacity in vitro, the maturational state of B-cells and the T-cell IFN-γ, IL-2 and IL-4 production in children with chronic renal failure.

PATIENTS AND METHODS

Eight children treated with peritoneal dialysis (PD), 8 children on chronic hemodialysis (HD) and 9 children with chronic renal failure, not yet dialyzed (CRF) were analyzed. The control group consisted of 9 healthy children (HC). The median (range) age, duration of PD or HD treatment and glomerular filtration rate (GFR) are listed in Table 1. The GFR in CRF patients was estimated by the Schwartz formula [22]. The primary renal disease of the patients is listed in Table 2. Four PD children were studied at 4 moments: before starting PD, and after 1,2 and 12 months of dialysis treatment (longitudinal data). Longitudinal samples were analyzed simultaneously. The study was approved by the
Medical Ethical Review Committee of the hospital and written informed consent was obtained from the children and/or parents.

**Table 1. Patient characteristics.**

<table>
<thead>
<tr>
<th></th>
<th>PD</th>
<th>HD</th>
<th>CRF</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Age</td>
<td>11.9 (4.5-15.3)</td>
<td>9.0 (1.7-16.7)</td>
<td>8.8 (4.9-18.4)</td>
<td>8.3 (5.4-16.3)</td>
</tr>
<tr>
<td>(years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of dialysis</td>
<td>4.6 (1.3-7.2)</td>
<td>1.6 (0.3-4.2)</td>
<td></td>
<td></td>
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<tr>
<td>(years)</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>GFR</td>
<td></td>
<td>14 (6-49)</td>
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<tr>
<td>(ml/min/1.73 m²)</td>
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</table>

GFR: glomerular filtration rate

**Table 2. Primary renal disease.**

<table>
<thead>
<tr>
<th></th>
<th>PD</th>
<th>HD</th>
<th>CRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urologic malformation</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Glomerulopathy</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Hemolytic uremic syndrome</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Metabolic disease</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Congenital disease</td>
<td>1</td>
<td>2</td>
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<tr>
<td>Other diseases</td>
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<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>


**Cell isolation**

Peripheral blood mononuclear cells (PBMC) were isolated from heparin blood by Ficoll-Isopaque density centrifugation. PBMC were frozen in 20% DMSO containing medium (75% wt/vol Earle's balanced salt solution, Tris buffered, 25% wt/vol fetal clone serum and penicillin/streptomycin) and stored in liquid nitrogen until use.
**B-cell phenotype assay**

PBMC were washed with PBAP (phosphate-buffered saline solution supplemented with 0.5% wt/vol bovine serum albumin, 0.01% wt/vol sodium azide and 0.5 mmol/L potassium-EDTA) and centrifugated (500 g, 10 min). Subsequently, cells were incubated with saturating amounts of CD2-FITC (Beckton Dickinson (BD) Immunocytometry Systems, San Jose, California), CD19-Percp (BD), CD5-FITC (BD), CD27-FITC (Sanquin blood supply foundation, CLB, Amsterdam), IgM-FITC (Pharmeringen, BD) and IgD-PE (Pharmeringen, BD) labelled monoclonal antibodies (moAb) for 30 minutes on ice in the dark. After incubation the cells were again washed with PBAP and centrifugated (500 g, 10 min). Flow cytometry analysis was performed directly thereafter with the FACS-Calibur (BD). The lymphocyte population was gated on the basis of forward-sideward scatter. B-lymphocytes were distinguished with anti-CD19 moAb. The percentage subset-positive or -negative B-lymphocytes were measured. A peripheral white blood cell count and differentiation was performed in all patients on a H3-Technicon counter (Technicon Instruments, Tarrytown, NY). The absolute count was calculated by the percentage subset-positive or -negative B-cells obtained from the flow cytometry multiplied by the absolute B-cell count. The absolute B-cell count was calculated by the percentage CD19+ B-cells multiplied by the absolute lymphocytes number.

**T-cell function assay**

This assay was performed with the BDIS FastImmune™ Cytokine System (BD) and is based on the detection of intracellular cytokines in activated lymphocytes [23;24]. PBMC were stimulated with PMA (Phorbol 12-Myristate 13 Acetate, final concentration 20 ng/mL), Ionomycin (final concentration 1 μg/mL) and BFA (Brefeldin-A, final concentration 10 μg/mL) (all from Sigma, St. Louis, MO). Cells were incubated for 4 hours at 37 °C, 7% CO2. This was followed by cell-surface staining with CD3-FITC, CD4-APC, CD8-Percp, leucogate and isotype-matched negative control moAb for 30 min on ice in the dark. Prior to the intracellular cytokine staining procedure cells were permeabilized with FACS Permeabilising Solution (BD) for 10 minutes in the dark. Thereafter, saturating amounts of PE-conjugated anti-IFN-γ, anti-IL-2 and anti-IL-4 moAb were added and incubated for 30 min at room temperature. Cells were fixed with Cellfix (BD). Flow cytometry analysis was performed on a FACS-Calibur (BD). The percentage cytokine producing lymphocytes and cytokine producing CD4+ or CD8+ T-cells were measured.

**In vitro Ig production assay**

Cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS), 0.1% β2-mercapto-ethanol, human transferrin 20 μg/mL and penicillin/streptomycin. Thereafter, PBMC were cultured (20,000 PBMC/well), unstimulated or stimulated under two different conditions: a combination of CD2-triple (CD2 triple, ascites; CLB-T11.1/1, final dilution 1:125, CLB-T11.2/1, final dilution 1: 125 and HIK27, final concentration 5 μg/mL) and CD28 (ascites, final dilution 1:1000) or a
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combination of SAC (Staphylococcal Aureus Cowan, final dilution 1:10000) and IL-2 (50 U/ml) for 8 days. Culture supernatant was collected and stored at –20 °C until use. IgG1, IgG2 and IgM content in supernatant were measured by ELISA [25].

Statistical analysis

The results are expressed as medians (range). Differences between the groups were tested with Kruskall-Wallis one-way analysis of variance. In case of significance, the Mann-Whitney-U test was performed to analyze differences between two groups. Longitudinal data were analyzed with a Friedman trend analysis and differences between two time points with the paired Wilcoxon test. Two sided p-values < 0.05 were considered significant.

RESULTS

B cell subset distribution

The medians (ranges) of the lymphocytes numbers and subsets are listed in Table 3, and the relative B-cell subset composition is depicted in Figure 1. The total lymphocyte count was lower in HD (P=0.015) and CRF (P=0.008) children compared with HC. The absolute CD19+ B-lymphocyte number was reduced only in HD children (P=0.04). The percentage IgM+ IgD- B-cells was not significantly different among all groups. However, there was a trend towards lower percentages IgM+ IgD- B-cells in PD, HD and CRF children compared to HC (Figure 1). The percentage (p=0.002) and the absolute number of CD27+ B-cells (p=0.016) were lower in PD children compared with HC. This was also found in HD children (P=0.027 and P=0.02) compared with HC. The absolute number of memory IgM+ IgD- B-cells was reduced only in HD children (P=0.046). No significant differences were found for children with CRF when compared to HC. CD5+ B-cells both, percentage and absolute number were lower in HD (P=0.046 and P=0.004) children compared with HC (Figure 1 and Table 3).

No significant changes of B-cell subsets occurred in the longitudinal analysis, during the first year of PD treatment (Figure 2, data shown only for IgM+ IgD- B-cells).

T helper cell functions

No differences were found in the proportion IFN-γ, IL-2 and IL-4 producing total lymphocytes numbers (data not shown). However, in the CD4+ T-cell population, HD children showed a significant lower percentage IL-2 producing cells compared with CRF children (Figure 3, P=0.027). CRF children had more IFN-γ producing cells in the CD8+ T-cell population compared with HC (Figure 3, P=0.015). Furthermore, HD children had a lower proportion IFN-γ producing CD8+ T-cells compared with CRF children (Figure 3, P=0.028). No differences were found in the T-cell IL-4 production between the groups (Figure 3).
Table 3. Phenotypic characteristics of CD19+ B-lymphocytes.

<table>
<thead>
<tr>
<th>Phenotypic Characteristic</th>
<th>PD</th>
<th>HD</th>
<th>CRF</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>2.8 (2.2-3.2)</td>
<td>1.9 (0.6-3.5)</td>
<td>2.0 (0.9-3.0)</td>
<td>2.8 (2.1-4.2)</td>
</tr>
<tr>
<td>CD19+</td>
<td>0.68 (0.13-0.97)</td>
<td>0.22 (0.07-1.33)</td>
<td>0.73 (0.07-2.09)</td>
<td>0.56 (0.35-0.96)</td>
</tr>
<tr>
<td>IgM/IgD+</td>
<td>0.08 (0.04-0.11)</td>
<td>0.05 (0.004-0.15)</td>
<td>0.04 (0.004-0.25)</td>
<td>0.09 (0.06-0.14)</td>
</tr>
<tr>
<td>CD27+</td>
<td>0.07 (0.03-0.13)</td>
<td>0.04 (0.01-0.15)</td>
<td>0.09 (0.02-0.29)</td>
<td>0.13 (0.06-0.28)</td>
</tr>
<tr>
<td>CD5+</td>
<td>0.21 (0.02-0.52)</td>
<td>0.03 (0.01-0.16)</td>
<td>0.11 (0.02-0.88)</td>
<td>0.18 (0.06-0.45)</td>
</tr>
</tbody>
</table>

Results are given in median (range) absolute number (x10⁶/L). PD, peritoneal dialysis; HD, hemodialysis; CRF, chronic renal failure, not yet dialyzed; HC, healthy controls. *P<0.05, **P<0.01.

Table 4. In vitro Ig production.

<table>
<thead>
<tr>
<th>IgG1</th>
<th>PD</th>
<th>HD</th>
<th>CRF</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstim</td>
<td>3.8 (1-17)</td>
<td>2.7 (1-9)</td>
<td>5.6 (3-39)</td>
<td>3.7 (1-13)</td>
</tr>
<tr>
<td>CD2/CD28</td>
<td>30 (19-110)</td>
<td>26 (1-55)</td>
<td>74 (1-602)</td>
<td>32 (1-263)</td>
</tr>
<tr>
<td>SAC/IL-2</td>
<td>0.2 (0.001-5.1)</td>
<td>0.2 (0.03-0.6)</td>
<td>0.1 (0.02-1.1)</td>
<td>0.2 (0.001-1.7)</td>
</tr>
<tr>
<td>IgG2</td>
<td>SAC/IL-2</td>
<td>&lt;1 (&lt;1-92)</td>
<td>&lt;1 (&lt;1-30)</td>
<td>&lt;1 (&lt;1-30)</td>
</tr>
<tr>
<td>Unstim</td>
<td>1.5 (1-30)</td>
<td>&lt;1 (&lt;1-9)</td>
<td>&lt;1 (&lt;1-12)</td>
<td>&lt;1 (&lt;1-7)</td>
</tr>
<tr>
<td>CD2/CD28</td>
<td>1.4 (0.2-15.0)</td>
<td>1.7 (0.03-4.5)</td>
<td>1.3 (0.07-12.6)</td>
<td>1.7 (0.06-5.4)</td>
</tr>
<tr>
<td>SAC/IL-2</td>
<td>8.6 (1-74)</td>
<td>3 (1-5)</td>
<td>2.1 (1-38)</td>
<td>1.4 (1-14)</td>
</tr>
</tbody>
</table>

Results are given in median (range). IgG, unstim and with anti-CD2/CD28 in ng/mL. IgG, with SAC/IL-2 in µg/mL IgM unstim in ng/mL. IgM CD2/CD28 and SAC/IL-2 stimulation in µg/mL. Unstim: unstimulated.

In the longitudinal analysis, during the first year of PD treatment no significant changes of cytokine production were present (Figure 4, data shown for IFN-γ in CD8+ T-cells and IL-2 in CD4+ T-cells).

In vitro Ig production

No significant differences were found in the in vitro IgG1, IgG2 or IgM production among all groups (Table 4). However, the range of Ig production was very wide. IgG2 production could only be detected in the cell cultures stimulated with SAC and IL-2.

In the longitudinal analysis, during the first year of PD treatment no alterations were found in the Ig production (Figure 5, data shown for IgG1).
Figure 1. The percentage of subsets of B-cells. PD, peritoneal dialysis; HD, hemodialysis; CRF, chronic renal failure; HC, healthy controls.

Figure 2. Longitudinal follow-up of the percentage IgM^+IgD^- B-lymphocytes during the first year of peritoneal dialysis (PD) treatment.
DISCUSSION

The present study has shown that dialysis treated children had lower numbers of IgM⁻/IgD⁻ and CD27⁺ B-lymphocytes, qualified as memory B-cells. Compared to healthy controls, the lowest numbers were found in HD children. However, the in vitro Ig production of PBMC was not different among all groups. Differences in T-helper cell cytokine secretion cannot explain the lower memory cell count since cytokine production was not significantly different between HD, PD and healthy children.

"Immunological memory" is important for a strong antibacterial and antiviral defence. B-cells differentiate from lymphoid stem cells into mature B-cells in the bone marrow, and through contact with antigen in secondary lymphoid tissues, they form germinal centers and undergo differentiation into memory cells or plasma cells [26]. IgM is expressed on immature B-cells. Both, IgM and IgD expression occur on mature B-cells. In the geriminal centers, B-cells undergo somatic hypermutation, isotype switching and affinity maturation. IgM and IgD expression is lost whereas IgG, IgA or IgE appear on the surface of plasma cells and memory B cells [27]. T-cells present in the germinal centers play an important role in this B-cell differentiation process by cross-linking of the CD40 molecule on the B-cell surface and CD40-ligand (CD40L) from T-cells [28,29]. Indeed, patients with hyper IgM syndrome, who have a mutation of CD40L, fail to produce IgG and IgA [30,31].

Both CD5 and CD27 were originally described as T-cell surface markers. In humans, CD5⁺ B-cells or naive B-cells are the first B-cells appearing in lymphoid tissues during early development and are present at very high frequency in the blood of newborns, diminishing through childhood and adulthood [32]. Furthermore, CD5 is expressed on the majority of peritoneal B-lymphocytes [33]. CD5⁺ B-cells secrete predominantly IgM [32]. Most CD5⁺ B-cells are found among IgM⁺IgD⁻ B-lymphocytes [34]. The hypothesis that PD children might have lower CD5⁺ B-cell count in blood because of peritoneal loss was not confirmed by our study. Similar results were found by Donze et al. in adult CAPD patients [33]. Only HD children had lower percentage and absolute number of CD5⁺ B-cells compared to HC.

CD27 is a marker for memory cells and is expressed on only a small proportion of B-cells, in contrast to T-cells [35,36]. The percentage CD27⁺ B-cells increases with age [8,9]. Low IgG production found in patients with X-linked hyper-IgM syndrome result from reduced numbers of IgD⁻CD27⁺ memory B-cells [37]. Data on memory B-cells are not yet available in uremic children and adults. Although the percentage IgM⁺IgD⁻ memory B-cells was not significantly lower in PD, HD and CRF children compared to HC, the lower absolute counts were present in PD and HD children. For the CRF group, significance was just not reached. A low IgM⁺IgD⁻ count could be the result of a low total lymphocyte number in our patients. However, the total lymphocyte count was not reduced in PD children. For CD27, both percentage and absolute number of positive B-cells were reduced, but again in CRF children the significance was not reached. Thus, dialysis patients have lower number of memory B-cells which might already be present before starting dialysis treatment. Possible explanations for this are a general suppression, sub-optimal T-helper activity or disturbances in B-cell migration process caused by uremia. Irrespective of the mechanism of memory B-
cell reduction, the consequence might be a lower capacity to mount a secondary immune response. Memory B-cells produce IgG earlier after exposure to antigen and have higher-affinity antigen receptors compared to unprimed B-cells. Further studies are needed to analyze the relation between B-cell subset differences in uremic patients and the lower IgG and subclasses levels as has been reported previously [5].

Figure 3. The proportion of cytokine (IFNγ, IL-2 and IL-4) producing T-helper (CD4+) and T-suppressor (CD8+) lymphocytes. PD, peritoneal dialysis; HD, hemodialysis; CRF, chronic renal failure; HC, healthy controls. *P<0.05.

In contrast to other studies [38,39], we found that PBMC, unstimulated and after stimulation with anti-CD2/CD28 moAb or SAC/IL2 produced equal amounts of IgG1, IgG2 and IgM in vitro compared to HC. The discrepancy between the studies can be explained by differences in methods such as stimulation conditions and patient number. Furthermore, the range of Ig levels was very wide and significant differences can only be found when a large number of patients is considered. In addition, since CD27+ B-cells are responsible for Ig production in vitro, it would be interesting to compare CD27+ B-cell numbers with Ig secretion in a larger number of patients. It should be noted however, that in the in vitro B-cell
differentiation assay interact with different cell types, cytokines and membrane receptors. Therefore, although dependent on the input of CD27⁺ B-cells, Ig secretion may not necessarily correlate with the subset in a quantitave way.

**Figure 4.** Longitudinal follow-up of the T-cell cytokine production in peritoneal dialysis (PD) children during the first year of dialysis treatment.

**Figure 5.** The *in vitro* IgG₁ production by lymphocytes from children during the first year of peritoneal dialysis (PD) treatment.

Interferon-γ is a cytokine released predominantly by CD4⁺ T cells of the Th1 type, CD8⁺ T cells and natural killer (NK) cells. This cytokine is involved in B-cell differentiation, Ig and subclass production and IgG subclass switch [12,13,40]. Furthermore IFN-γ promotes the activity of antigen presenting cells, and maintains and enhances the functional properties of phagocytes. IFN-γ treatment in patients with chronic granulomatous disease decreases the frequencies of serious infections [41]. Lower amount of IFN-γ released by peritoneal lymphocytes from PD patients with high peritonitis incidence in comparison with patients
with low peritonitis incidence and healthy persons has been reported by Lamperi et al.[42]. The bacterial killing of peritoneal macrophages improved after treatment with IFN-γ. Lu et al. demonstrated that IFN-γ levels in peritoneal dialysis effluent of PD patients with peritonitis could only be detected during Staphylococcus aureus or Staphylococcus epidermidis peritonitis. Serum levels were below the detection limit [43]. The toxicity of peritoneal dialysis solutions on immunological function of peritoneal cells, might reduce the production of cytokines by peritoneal lymphocytes during PD. We found no significant differences in IFN-γ production by peripheral blood T-lymphocytes in CRF, PD and HD children but we did not measure the cytokine production of peritoneal lymphocytes. We could not confirm the Th1/Th2 imbalance, previously reported in patients treated with dialysis [17]. Differences in methodology, age of the patients and low patient number are possible explanations for this discrepancy.

In conclusion, children with chronic renal failure, especially those treated with dialysis, have lower numbers of memory B-cells. This reduction may contribute to the low Ig levels found in these children.

REFERENCES


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