Functional and immunological studies in children with chronic renal failure: the effects of uremia and dialysis treatment
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Chapter 8

**Fcγ and complement receptor expression on peripheral white blood cells in uremic children.**

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Submitted
**ABSTRACT**

Phagocytosis of IgG or complement opsonized bacteria, and the antibody production by lymphocytes are regulated by cell surface receptors for IgG (FcγRI, FcγRII and FcγRIII) and complement (CR1 and CR3). We measured the effect of uremia and dialysis treatment modalities on FcγR and CR expression on leukocytes in blood of forty children treated with peritoneal dialysis (PD), 23 on hemodialysis (HD), 46 not yet dialyzed (CRF) and 33 healthy children (HC).

White blood cells (WBC) were isolated from EDTA-blood by centrifugation after cell fixation with paraformaldehyde. Subsequently, WBC were labelled with FITC-conjugated CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγRI), CD11b (CR3), and CD35 (CR1) monoclonal antibodies and analyzed by flow cytometry.

In PD, HD, CRF and HC, monocytes and neutrophils were all positive for FcγR and CR except CD16 on monocytes (20% positive). The median CD32 MFI on lymphocytes, monocytes, and neutrophils was lower in PD (33, 54 and 47), HD (33, 55 and 53) and CRF children (35, 52 and 50) compared with HC (156, 77 and 69, $P<0.01$). On the other hand, CD11b MFI on lymphocytes, monocytes and neutrophils was higher in PD (19, 36 and 43), HD (26, 39 and 42), and CRF children (27, 39 and 44) compared with HC (15, 28 and 25, $P<0.05$). CD16 and CD64 MFI were not different among the groups and CD35 MFI was only lower on lymphocytes from PD (30), HD (30) and CRF (31) children compared with HC (47, $P<0.05$).

In children with chronic renal failure, dialyzed or not, the FcγRIII expression on lymphocytes, monocytes and neutrophils was reduced, combined with an increase of the CR3 expression. These abnormalities are attributed to the uremic state and might contribute to an impaired immune function.
INTRODUCTION

Fc and complement receptors are important for the interaction between the humoral and cellular immunity. These receptors bind to IgG and complement opsonized microorganisms and facilitate the phagocytic process. Fc receptors for IgG, also called FcγR, can be divided in three different types designated FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), each with unique binding characteristics for IgG and its subclasses [1,2]. FcγR are present on monocytes, neutrophils, NK cells and lymphocytes.

Four different complement receptors are known, CR1, CR2, CR3 and CR4. CR1(CD35), an opsonic receptor for C3b, is present on neutrophils and monocytes mediating phagocytosis. CR1 on B lymphocytes, together with CR2, mediates lymphocyte activation. Cleavage of C3b by factors H and I results in an inactive complement protein iC3b which has a low affinity for CR1. However, iC3b still has an important opsonic activity by binding also additionally to CR3 (CD11b) and CR4. CR3 and CR4 augment the activities of Fc receptors and CR1 in activating phagocytosis [3-5]. CR1 and CR3 are especially important for inducing the phagocytosis of complement-coated bacteria.

Little information is available on the FcγR and CR expression or function in blood of patients with chronic renal failure, treated with dialysis or not. Some authors described an increased CD16 (FcγRIII) positive monocyte population in PD and HD patients when compared to healthy controls, a phenotype that has been linked to tissue macrophages in the context of the stage of maturation [6,7]. No difference was found by Braun et al. in the CD11b (CR3) expression on monocytes between adult PD patients and healthy controls. Conflicting reports are present on the macrophage FcγR function in adult patients with end stage renal disease [8,9]. Ruiz et al. [9] described an impaired macrophage FcγR function in HD patients, whereas Halma et al. [8] could not confirm this, probably because of differences in the methods they used. No information is available on the expression of FcγRII (CD32) and FcγRI (CD64) on white blood cells of stable PD and HD patients. In children on PD, a longitudinal study (3 years) has been performed by Wasik et al. on CD16 (FcγRIII) and CD35 (CR1) expression on phagocytic cells. They found a temporary increase of the percentage CD16 and CD35 positive neutrophils in blood, during the first 3 months of PD treatment [10].

In order to further explore the mechanisms responsible for the immune system abnormalities during chronic renal failure, and the influence of dialysis treatment on this, the FcγR and CR expression of white blood cells were analyzed in children with chronic renal failure not yet dialyzed, and in children treated with hemodialysis or peritoneal dialysis.

PATIENTS AND METHODS

Blood samples were obtained for FcγR and CR expression analysis from 40 children treated with PD, 23 children on hemodialysis (HD), 46 children with chronic renal failure not yet dialyzed (CRF) and 33 healthy control children (HC). Blood samples were drawn just before starting the hemodialysis session in HD children. The medians (ranges) of
age, glomerular filtration rate (GFR) and duration of dialysis treatment are summarized in Table 1. In CRF patients, the GFR was estimated by the Schwartz formula [11]. The primary renal disease of the patients is listed in Table 2. Thirty-three percent (15/46) had a moderate renal insufficiency (25 to 50 ml/min/1.73 m²), 30% (14/46) a severe renal insufficiency (10 to 25 ml/min/1.73 m²) and 37% (17/46) preterminal renal failure (≤ 10 ml/min/1.73 m²).

Table 1. Patients 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>40</td>
<td>23</td>
<td>46</td>
<td>33</td>
</tr>
<tr>
<td>Age (years)</td>
<td>10.1 (1.7-18.3)</td>
<td>13 (1.7-19.2)</td>
<td>10.3 (0.5-19.9)</td>
<td>6.0 (0.8-17.5)</td>
</tr>
<tr>
<td>Duration of dialysis (years)</td>
<td>1.4 (0.2-8.9)</td>
<td>2.2 (0.4-7.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFR (ml/min/1.73 m²)</td>
<td></td>
<td>15 (4-50)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are given in median (range). PD: peritoneal dialysis, HD: hemodialysis, CRF: chronic renal failure, HC: healthy controls. 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>13</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Glomerulopathy</td>
<td>10</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Hemolytic uremic syndrome</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Metabolic disease</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Congenital disease</td>
<td>7</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Other diseases</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>23</td>
<td>46</td>
</tr>
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</table>


Blood was sampled by venapuncture in EDTA-tubes. White blood cells (WBC) were isolated by centrifugation (500 g, 10 min, 4°C) after cell fixation with paraformaldehyde 1% (PFA). Erythrocytes were lysed with ammonium chloride (0.155 mol/L) and potassium-EDTA (0.5 mmol/L) and WBC were subsequently 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). WBC were again fixated with 4% PFA for 10 minutes.
followed by centrifugation (500 g, 10 min, 4°C). The Fc receptor of WBC was blocked with 10% normal human pool serum (NPS). WBC were incubated with saturating amounts of FITC labeled CD16 (FcγRIII) obtained from the CLB Sanquin Blood Supply Foundation, Amsterdam (CLB, clone CLB-Fc-gran/1, 5D2), CD32 (FcγRII) (Instruchemie Hilversum B.V., clone AT10), CD64 (FcγRI) (Medarex, Annandale, clone P3/NS1/1-Ag4-1), CD11b (CR3) (CLB, clone CLB-mon-gran/1, B2), and CD35 (CR1) (Instruchemie, clone E11), monoclonal antibodies (moAb) for 30 minutes on ice in the dark. After incubation the cells were washed with PBAP and again fixated with PFA 1%. Flow cytometry analysis was performed within 12 hours thereafter with the FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, California, USA). Lymphocytes, monocytes and neutrophils were distinguished on the basis of their size and granularity by using a dot plot of forward scatter (FSC) versus side scatter (SSC). To adjust the monocyte population, PE-conjugated anti-CD14 moAb was used and for the neutrophils anti-CD16 was used. Peripheral blood mononuclear cells (PBMC) from a buffy coat of a healthy person were included in every FACS analysis for the inter-assay variations. A total of 20,000 events were measured for each leukocyte sample. The positively labeled fraction was determined from the comparison with an isotype matched control Ab. The percentage FcγR- and CR- positive cells was calculated. The number of receptors per cell was expressed as mean fluorescence intensity, MFI.

The study was approved by the Medical Ethical Review Committee of the hospital and written informed consent was obtained from children and/or parents.

**Statistical analysis**

The results are expressed as medians and ranges. Differences between all groups were tested with Kruskall-Wallis one-way analysis of variance. Differences between two groups were tested with the non-parametric unpaired Mann-Whitney test.

**RESULTS**

**Fcγ-receptors**

CD16 (FcγRIII). Lymphocytes and neutrophils were all positive for CD16, whereas only 20% of the monocytes were positive for CD16. The percentage CD16 positive monocytes was slightly lower in CRF children (median 18, range 3 to 35) compared with HC (median 22, range 7 to 36, P<0.01). No differences were found between PD, HD and CRF children. The CD16 MFI was not different among the groups (Table 3).

CD32 (FcγRII). The median percentage CD32 positive lymphocytes was 21 (range 11 to 34) in HC compared with 15 (range 5 to 32) in PD (P<0.001), 14 (range 7 to 33) in HD (P<0.0001) and 16 (range 5 to 34) in CRF (P=0.001) patients (Figure 1). The CD32 MFI was lower in PD, HD and CRF children compared with HC (P<0.01, Table 3)

CD64 (FcγRI). The expression of CD64 on neutrophils was very low and was not different among the groups (Table 3). Also, the expression on monocytes was not different.
Table 3. The FcγR and CR expression (MFI) on white blood cells.

<table>
<thead>
<tr>
<th></th>
<th>PD</th>
<th>HD</th>
<th>CRF</th>
<th>HC</th>
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</thead>
<tbody>
<tr>
<td>CD32</td>
<td>Ly: 33 (11-245)</td>
<td>Mo: 54 (36-141)</td>
<td>Neu: 47 (30-112)</td>
<td>156 (21-905)</td>
</tr>
<tr>
<td>CD64</td>
<td>Mo: 51 (27-105)</td>
<td>Neu: 14 (9-58)</td>
<td></td>
<td>50 (14-92)</td>
</tr>
<tr>
<td>CD35</td>
<td>Ly: 30 (6-62)</td>
<td>Mo: 14 (9-113)</td>
<td>Neu: 17 (8-56)</td>
<td>47 (18-137)</td>
</tr>
</tbody>
</table>

PD: peritoneal dialysis, HD: hemodialysis, CRF: chronic renal failure, HC: healthy controls. Ly: lymphocytes, Mo: monocytes, Neu: neutrophils, NK: NK cells. *p<0.05, **p<0.01, ***p<0.001,  p=0.057 when compared with HC.

**Complement-receptors**

CD11b (CR3). The percentage CD11b positive lymphocytes was 20% (range 4 to 40) in PD children (P<0.01), 28% (7 to 50) in HD children (NS), 24% (range 7 to 71) in CRF patients (NS) compared with 27% in HC (range 13 to 52). PD children had a lower percentage CD11b positive lymphocytes than HD and CRF children (P<0.01). The MFI of CD11b on lymphocytes was higher in PD (P<0.01), HD (P<0.05) and CRF (P<0.001) patients compared with HC (Table 3). The CD11b expression on monocytes was higher in HD (P<0.05) and CRF (P<0.001) children compared to HC (Table 3). For the PD group, this was just not significant (p=0.057). The MFI of CD11b on neutrophils was also higher in PD (P<0.001), HD (P<0.05) and CRF (P<0.001) children compared with HC (Table 3).

CD35 (CR1). The percentage CD35 positive lymphocytes was 20% (range 4 to 36) in PD children (NS), 15% (range 6 to 36) in HD children (P<0.0001), 19% (6 to 41) in CRF patients (P<0.05) compared with 24% (10 to 42) in HC (Figure 2). The MFI of CD35 on lymphocytes was lower in PD (P<0.001), HD (P<0.05) and CRF (P<0.001) children compared with HC (Table 3). No differences were found in the CD35 MFI on monocytes between the groups. The MFI of CD35 on neutrophils was only lower in CRF children compared to HC (P<0.01, Table 3). Furthermore, CRF children had a slightly lower CD35 expression on neutrophils compared with HD children (P=0.04, Table 3).
**DISCUSSION**

Possible differences of FcγR and CR expression on white blood cells between children with chronic renal failure, dialyzed or not, and their healthy controls have been investigated in the present study. Differences were found especially for FcγRII (CD32) and CR3 (CD11b). The percentage CD32 positive lymphocytes, monocytes and neutrophils, and CD32 MFI was lower in PD, HD and CRF children in comparison to HC. On the other hand CD11b MFI, but not the percentage positive cells, was higher on lymphocytes, monocytes and neutrophils in all patient groups compared with HC. Consequently, these findings are related to the presence of chronic renal failure itself.

It is well established that binding of opsonized particles to FcγR and CR enhances the efficiency of the phagocytic process [12]. Children treated with peritoneal dialysis (PD) were found to have a higher peritonitis incidence than adult PD patients [13], but the function of FcγR or CR was not analyzed. The exact mechanism behind the increased susceptibility to infections in dialysis patients has still not been elucidated. The lower FcγRII expression on monocytes and neutrophils, might result in a reduced phagocytic capacity and thus an increased susceptibility to infections. This assumption is strengthened by a study from Rossmann et al. who found a positive correlation between macrophage FcγRII expression and FcγR function [14].

CR3 is not competent to mediate phagocytosis in resting unactivated phagocytes, but this receptor is activated after the phagocyte is exposed to cytokines and
Children with leukocyte adhesion deficiency (LAD) have a complete or almost complete absence of this receptor, which leads to uncontrolled tissue infection and delay of wound healing [16]. Zhou et al. have demonstrated a functional and physical association of FcγRII with CR3 [17]. The increased CR3 expression in our patients might be a reflection of chronic inflammation caused by the state of chronic renal failure. The consequence of the combination of a lower FcγRII and a higher CR3 expression in children with renal failure remains unclear.

The influence of lymphocyte FcγR and CR expression on the regulation of antibody production in humans is not completely understood. One study from Takai et al. performed in mice reported a negative feedback mechanism between the FcγRII expression and antibody production [18]. If this were the case in the population of the present study, high IgG levels would have been expected. However, we previously reported the presence of low IgG2 levels in the same population of children with chronic renal failure [19]. This may be relevant, because the allotype FcγRIIa-H131 of FcγRII, is the only receptor that can bind IgG2 [20]. Reduced serum IgG2 levels have been reported in children with CRF [19]. Reduced FcγRII expression and low IgG2 serum levels together might thus result in a reduced capacity to kill microorganisms, for example pneumococcal encapsulated bacteria.

It has been demonstrated that treatment with granulocyte-macrophage colony stimulating factor (GM-CSF) in children with solid tumors results in a decline of the FcγRII and FcγRIII expression on neutrophils combined with an increase of CR3 [21]. However, an increase of the FcγRII expression was found on monocytes. Only one study in adult PD patients reported increased plasma GM-CSF levels [22]. Thus, it might be that the reduced FcγRII combined with the increased CR3 expression found in our study could be caused by increased GM-CSF plasma levels. No information is available on plasma GM-CSF levels in children with chronic renal failure.

The expression of FcγR and CR on WBC is regulated by cytokines [1,12,23,24]. Many studies have been performed on cytokine production in the peritoneal dialysis effluent in relation to peritonitis [25-28] and in blood of HD patients for analysis of the biocompatibility of the dialyzer membrane [29,30]. Interferon-γ (IFN-γ) is an important cytokine for bacterial killing through macrophages. IFN-γ treatment increased the FcγRI expression on monocytes from newborns and adults, but the FcγRIII expression on monocytes increased only in newborns, but not in adults [31]. Erbe et al. demonstrated an upregulation of only FcγRI on monocytes and neutrophils in vitro after IFN-γ treatment [24]. Differences in cytokine production by lymphocytes and monocytes might play a role in the alterations of FcγR and CR expression on WBC found. In the present study however, data on such relationships are not available.

The expression of the other Fcγ-receptors was not different among the groups, except a slightly lower FcγRIII- (CD16) positive monocyte population, in CRF children when compared with HC. This is in contrast to other studies performed in adult PD patients that described a higher CD16 positive monocyte population. CD16 appears on the cell surface of
monocytes during their maturational process. This implicates that monocytes from children with CRF are less mature compared to their healthy controls and possibly also when compared to adult patients with CRF. For CR1 (CD35) only the lymphocytes of patient groups showed a lower percentage CD35 positive cells combined with a lower MFI than their HC. This might also influence the antibody production by B-cells.

In summary, children with CRF, dialyzed or not, showed a down-regulation of FcγRII (CD32) on lymphocytes, monocytes and neutrophils, combined with an upregulation of CR3 (CD11b) expression levels. It is speculative whether these alterations might result in a diminished effectiveness of the immune system.

REFERENCES


