Functional and immunological studies in children with chronic renal failure: the effects of uremia and dialysis treatment
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Chapter 7

Effects of peritoneal dialysis treatment and peritonitis on IgG and complement receptor expression on phagocytes in children.

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Submitted
ABSTRACT

Children treated with peritoneal dialysis are at increased risk of infections. IgG receptors (FcγRI, FcγRII, and FcγRIII) and complement receptors (CR1 and CR3) on phagocytic cells are important for the phagocytic process. We have investigated the FcγR and CR expression on monocytes, macrophages and neutrophils in blood and in peritoneal dialysis effluent (PDE) of 39 children treated with peritoneal dialysis.

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

Results: Peritoneal cells had lower percentages of FcγR or CR positive cells compared to blood. On the other hand, the receptor numbers per cell (MFI) were higher on peritoneal macrophages and neutrophils compared to blood except for CD16. FcγR and CR expressions in blood and dialysate did not change significantly during the first year of PD treatment. During a peritonitis episode, the MFI of all receptors in blood increased only on monocytes with exception of CD32. The percentage FcγR and CR positive macrophages and neutrophils in the PDE increased, whereas MFI did not.

Conclusion: Peritoneal cells of PD children showed a lower percentage FcγR and CR positive neutrophils and macrophages combined with an increased MFI, indicating a state of activation. Blood and peritoneal cells are capable of up-regulating the receptor expression during peritonitis but probably not to a maximum level.
INTRODUCTION

Monocytes, macrophages and neutrophils are important for the phagocytosis of microorganisms. The phagocytic function in uremic patients is reduced [1]. Peritonitis is still a major problem in some children treated with peritoneal dialysis [2-4]. This results in a higher morbidity and treatment failure. Receptors for the constant region of IgG (FcγR) and for complement (CR) on phagocytic cells bind to antibody-, or complement-opsonized microorganisms and facilitate phagocytosis [5]. Three classes of receptors for IgG exist on leukocytes: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). They differ in their structure, binding affinity for IgG, cell distribution and downstream signalling [6,7]. CR1 (CD35) and CR3 (CD11b) represent CR present on monocytes/macrophages and neutrophils. Activated complement components such as C3b, bind to CR of leukocytes for the mediation of several mechanisms such as uptake of particles opsonized by complement or activation of the cell [5]. Reduced numbers of FcγR and CR on peritoneal macrophages have been shown in adult CAPD patients with prolonged duration of PD treatment [8]. On the other hand, CAPD children showed increases in the percentage FcγR and CR positive peritoneal macrophages shortly after starting PD treatment [9]. Brauner et al. described a higher expression of CD11b and CD16 on peritoneal macrophages compared with blood monocytes [10]. No information is available on the comparison of FcγR and CR expression on leukocytes in blood and dialysate of PD children in a stable situation and during periods of infections.

The aim of the present study was to analyze expression of FcγR and CR on monocytes and neutrophils in blood and peritoneal dialysis effluent of stable PD children. Fifteen of them were followed during the first year of PD treatment, and nine were also studied during the acute phase of a peritonitis episode and during follow-up.

PATIENTS AND METHODS

Blood and dialysate samples were obtained for analysis of CR and FcγR expression on monocytes / macrophages and neutrophils from 39 children treated with PD. Demographic data, primary renal disease and peritonitis incidence are given in Table 1. Fifteen children were followed from before starting PD during the 12 months thereafter (at 1,2,3,6 and 12 months). Nine children could be analyzed during a peritonitis episode, on the day of admittance to the hospital (P0), after 14 (P14) and 28 (P28) days and in a stable situation, more than 3 months before or after the peritonitis episode. The peritonitis was caused by a gram-negative microorganism in 4 children and a gram-positive microorganism in 5 children.

White blood cells (WBC) were isolated from EDTA-blood 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 (0.14 M Sodium-chloride, 10 mM sodium-phosphate, supplemented with 0.5% wt/vol bovine serum albumin, 0.01% wt/vol sodium azide and 0.5
### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Patients</th>
<th>39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>10.3 (1.7-18.3)</td>
</tr>
<tr>
<td>Duration of dialysis (years)</td>
<td>1.4 (0.1-8.9)</td>
</tr>
<tr>
<td>Peritonitis incidence (episodes/year)</td>
<td>0.8 (0-7.9)</td>
</tr>
<tr>
<td>Primary renal disease (%)</td>
<td></td>
</tr>
<tr>
<td>Urologic malformation</td>
<td>13 (33.3)</td>
</tr>
<tr>
<td>Glomerulopathy</td>
<td>10 (25.6)</td>
</tr>
<tr>
<td>Hemolytic uremic syndrome</td>
<td>4 (10.3)</td>
</tr>
<tr>
<td>Metabolic disease</td>
<td>1 (2.6)</td>
</tr>
<tr>
<td>Congenital disease</td>
<td>6 (15.4)</td>
</tr>
<tr>
<td>Other diseases</td>
<td>3 (7.7)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (5.1)</td>
</tr>
</tbody>
</table>

Age, duration of dialysis and peritonitis incidence are given in median (range) values. Primary renal disease is given in number of patients and percentage (%). Urologic malformation: 8 children with urethral valves, 4 reflux nephropathy, 1 bilateral duplicated kidney. Glomerulopathy: 5 children with focal segmental glomerulosclerosis, 2 congenital nephrotic syndrome, 1 rapidly progressive glomerulonephritis, 1 alport syndrome, 1 Henoch Schönlein-purpura nephritis. Congenital disease: 3 children with renal dysplasia, 1 polycystic kidney disease, 1 nephropathia, 1 tuberous sclerosis. Other diseases: 2 children with acute tubular necrosis, 1 bilateral Wilms tumor.

mmol/L potassium-EDTA). WBC were again fixated with 4% PFA for 10 minutes followed by centrifugation (500 g, 10 min, 4°C). Fc receptors on WBC were blocked with 10% normal human pool serum (NPS). Peritoneal cells from the peritoneal dialysis effluent (PDE) of a four hour dwell using a 1.36% glucose solution (Dianeal®, Baxter BV, Utrecht, The Netherlands) were isolated by centrifugation (500 g, 10 min, 4°C). This was followed by fixation with PFA 4%. After centrifugation (500 g, 10 min, 4°C), the Fc receptor of peritoneal cells was blocked with 10% NPS. WBC and peritoneal cells were incubated with saturating amounts of FITC- labelled CD11b (CR3) obtained from the CLB Sanquin blood supply foundation, Amsterdam (CLB, clone CLB-mon-gran/1, B2), CD35 (CR1) (Instruchemie, clone E11), CD16 (FcγRIII) (CLB,clone CLB-Fc-gran/1, 5D2), CD32 (FcγRII) (Instruchemie Hilversum B.V., clone AT10), and CD64 (FcγRI) (Medarex, Annandale, clone 32.2) monoclonal antibodies (mAb) for 30 minutes on ice in the dark. Isotype-matched control antibodies were used to define cut offs for positively labeled cells. After incubation cells were washed with PBAP and again fixated with PFA 1%. Flowcytometry was performed within 12 hours thereafter on a FACSScan (Beckton Dickinson, San Jose, CA). 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, or macrophage populations in, respectively, blood or PDE, PE-conjugated anti-CD14 mAb (BD) 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 all FACS analyses to
control for inter-assay variation. A total of 20,000 events were measured for each leukocyte sample. Positively labeled cell fractions were determined by comparison with isotype matched inert antibodies. The percentage CR- or FcγR-positive cells was calculated. The number of receptors per cell or receptor density of the receptor positive population is expressed as mean fluorescence intensity or MFI.

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

**Statistical analyses**

Results are expressed as median and range. Differences between blood and dialysate were tested with non-parametric matched pair Wilcoxon tests. Longitudinal data were analyzed with Friedman trend analyses and differences between two time points with paired Wilcoxon tests. Correlations were analyzed with Spearman rank correlation tests.

**RESULTS**

**Cross sectional analyses**

The percentages of cells that were positive for CR or FcγR are listed in Table 2. More than 90% of blood monocytes and neutrophils were positive for all receptors with the exception of CD16 on monocytes, which was present on 20% of cells. In contrast, significantly less peritoneal macrophages and neutrophils expressed CR or FcγR, although the range of receptor-positive cells in dialysates was wide. The MFI of CD11b was higher on peritoneal macrophages (141) and neutrophils (126) compared to blood monocytes (38) and neutrophils (44). The MFI of CD35 was also higher on peritoneal macrophages (21) and neutrophils (63) compared to blood monocytes (14) and neutrophils (18) (Fig. 1a). This was also found for CD32; 106 on macrophages versus 54 on monocytes, and 99 on peritoneal neutrophils versus 47 on blood neutrophils. For CD64, only peritoneal neutrophils showed a higher MFI (47) compared with blood (14) (Fig. 1b). CD16 MFI were lower on peritoneal macrophages (22) compared with blood monocytes (45), and not different for neutrophils (153 versus 136) (Fig. 1b).

No relation could be found between the duration of PD treatment and CR or FcγR expression levels, except for the CD35 MFI on peritoneal neutrophils (r=0.5, 95% CI: 0.2 - 0.7, p=0.003).

**Longitudinal analyses**

In blood, all children had at least 3 time points for analysis, whereas in dialysate 3 or more time points could be analysed in 9 children. During the first year of PD treatment no significant changes were noted in CR or FcγR expression in blood or dialysate (Fig. 2, data shown only for CD35).
Table 2. Percentage CR and FcγR positive cells.

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Cells</th>
<th>Blood</th>
<th>PDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b (CR3)</td>
<td>Monocytes/macrophages</td>
<td>94 (76-98)</td>
<td>86 (43-99)</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>99 (93-100)</td>
<td>75 (2-99)</td>
</tr>
<tr>
<td>CD35 (CR1)</td>
<td>Monocytes/macrophages</td>
<td>97 (89-100)</td>
<td>50 (11-91)</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>99 (94-100)</td>
<td>49 (0-88)</td>
</tr>
<tr>
<td>CD16 (FcγRII)</td>
<td>Monocytes/macrophages</td>
<td>20 (12-39)</td>
<td>40 (1-80)</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>98 (92-100)</td>
<td>13 (1-85)</td>
</tr>
<tr>
<td>CD32 (FcγRII)</td>
<td>Monocytes/macrophages</td>
<td>96 (86-100)</td>
<td>91 (42-100)</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>99 (93-100)</td>
<td>51 (1-96)</td>
</tr>
<tr>
<td>CD64 (FcγRI)</td>
<td>Monocytes/macrophages</td>
<td>98 (67-100)</td>
<td>92 (53-99)</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>98 (91-100)</td>
<td>51 (1-100)</td>
</tr>
</tbody>
</table>

Results are given in median values and range. MoAb: monoclonal antibody PDE: peritoneal dialysis effluent. The difference between peripheral blood and peritoneal dialysis effluent was statistically significant for all receptors (p ≤ 0.003).

Peritonitis

The percentage CD16 positive monocytes in blood did not increase during peritonitis (from 19%, range 10-30% to 19%, range 11-39%). All other receptors were already stably present on the majority of cells. The percentage of peritoneal macrophages positive for CR or FcγR increased to 100%, with the exception of CD16 on macrophages which changed from 6% to 24%. However, this change did not reach significance (p=0.08). The percentage CR- or FcγR- positive neutrophils in PDE increased to around 100%. The results on MFI are expressed as relative increase / decrease in MFI at the time of peritonitis.

Figure 1a. Expression of CR on blood (bl) and peritoneal (pde) monocytes/macrophages and neutrophils (mean fluorescence intensity or MFI).
presentation (P0), relative to the MFI in a stable situation (Table 3). In blood, the MFI of CR and FcγR on monocytes increased, with exception of CD32. The MFI of CR and FcγR on neutrophils in blood did not change significantly. In dialysate, the MFI of CD35 and CD64 on macrophages increased. For CD16 on macrophages statistical significance was just not reached (p=0.08). The MFI of the other receptors showed no significant change. Peritoneal neutrophils did not show an increase of the CR or FcγR MFI. CD64 MFI on peritoneal neutrophils decreased.

To assess whether differences existed in CR and FcγR expression between a gram-positive or a gram-negative peritonitis we compared the relative changes of CR and FcγR during peritonitis in these two groups. No significant differences were found between peritonitis caused by gram-positive or gram-negative microorganisms. The CR and FcγR expression on phagocytes in blood and dialysate of children with a peritonitis incidence > 1 episode per year (high peritonitis incidence) was not different from those with < 1 peritonitis episode (low peritonitis incidence) per year.
Table 3. Relative changes of CR and FcγR density (MFI) on phagocytes during peritonitis.

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>PDE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>monocytes</td>
<td>neutrophils</td>
</tr>
<tr>
<td>CD11b</td>
<td>1.9 (0.7-2.2)</td>
<td>0.9 (0.6-2.0)</td>
</tr>
<tr>
<td>CD35</td>
<td>2.2 (0.7-9.3)</td>
<td>1.0 (0.3-4.4)</td>
</tr>
<tr>
<td>CD16</td>
<td>2.0 (0.7-5.2)</td>
<td>1.0 (0.7-4.9)</td>
</tr>
<tr>
<td>CD32</td>
<td>1.4 (0.6-2.9)</td>
<td>1.0 (0.4-1.5)</td>
</tr>
<tr>
<td>CD64</td>
<td>1.6 (0.9-10.7)</td>
<td>1.1 (0.1-2.6)</td>
</tr>
</tbody>
</table>

Results are given in median relative increase/decrease and range. The relative change is calculated by the MFI of cells at peritonitis presentation (P0) divided by the MFI in a stable situation. p<0.05, **p<0.01, *p=0.08.

DISCUSSION

Macrophages and neutrophils in the peritoneal dialysis effluent showed a lower proportion FcγR- and CR- positive cells compared to WBC. However, peritoneal cells showed an increased expression density (MFI), reflecting a state of activation. Except for CD32, monocytes and macrophages showed an upregulation of their receptor density during a peritonitis episode; neutrophils in blood and PDE were unable to create a further upregulation of their MFI.

FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) are present on monocytes, macrophages and neutrophils and the expression is linked to their state of activation [6]. Only FcγRIII cell surface expression levels diminish after stimulation, because of receptor internalization and (soluble) shedding of the soluble receptor from the cell surface [11]. When FcγR and CR on monocytes or neutrophils are cross-linked by IgG or complement opsonized particles, several immunological effector functions can be established such as phagocytosis of IgG-coated bacteria, erythrocytes, platelets, leukocytes or tumor cells, clearance of immune complexes, release of inflammatory mediators, and antibody dependent cellular cytotoxicity [6,12]. CR1 primarily promotes the adhesion of particles and CR3 mediates the subsequent engulfment [13].

Our findings of an increased FcγR and CR expression on peritoneal macrophages and neutrophils would suggest that their phagocytic function is better, although this might result in an early exhaustion. On the other hand peritoneal cells showed also a substantial FcγR- and CR- negative population in contrast to blood. This was especially clear for neutrophils. Bacterial killing by FcγR- or CR- receptor negative cells is
Figure 2. Longitudinal analysis of CD35 (CD1) expression on blood monocytes and peritoneal macrophages during the first year of PD treatment.

much slower [13,14]. It has been reported that peritoneal cells, especially macrophages are relatively immature cells with signs of activation [15,17]. However, in the present study these Fcγ- or CR-negative cells are probably not relatively young, immature cells related to the high turnover through frequent bag exchanges, because peritoneal cells are derived from the blood where the majority of the cells is Fcγ- or CR-positive. Moreover, during peritonitis, when the turnover of peritoneal cells is even higher, the percentage Fcγ- or CR positive cells showed an increase. Thus, other factors are likely to be involved, such as the migration process of WBC from the blood circulation into the peritoneal cavity, or the toxicity of the dialysate affecting the expression of FcγR and CR.

Few studies have been performed on macrophage FcγR function in patients with end-stage renal disease. Some authors demonstrated an impaired macrophage FcγR function in HD or PD patients [18,19] whereas Halma et al. showed a normal FcγR function [20]. This discrepancy is probably caused by methodological differences in these studies. It has been reported that the peritoneal macrophages, isolated from 3.86% dextrose effluent, showed a lower phagocytic capacity than macrophages obtained from a 1.36% dextrose concentration [21]. In the present study, a standard dwell with glucose 1.36% was used. The relation between the usual glucose concentration of the child and the FcγR and CR expression levels was tested in 19 children. No differences were found between the usual glucose concentration and receptor expression levels (unpublished data).

Carcamo et al. showed a decrease of FcγR and CR expression on macrophages in PD patients with prolonged duration of PD treatment [8]. A longitudinal analysis on neutrophil receptor expression was not performed in that study. In our cross sectional analysis, we found a positive relationship between level of expression and duration of PD treatment only for CD35 (CR1) on peritoneal neutrophils. This might reflect a state of activation. The reason why for CR3 (CD11b) no positive correlation has been found should
be bound to different physiological responses of these receptors to a certain stimulus, which has been reported by others [22,23]. However, in longitudinal analyses no alterations of any FcγR or CR expression levels were found during the first year of PD treatment. A minority of children displayed an increase or decrease of the CD35 MFI on peritoneal neutrophils during the first year of PD treatment (Figure 2). These children had no peritonitis episode around these measurements. Maybe a subclinical bacterial contamination in the peritoneal cavity resulted in these alterations.

Alterations in FcγR and CR levels on neutrophils in febrile “healthy” individuals during bacterial and viral infections have been studied by Leino et al. [23]. They found a significant increase in expression of CD64 (FcγRI), CD32 (FcγRII) and CD35 (CR1), but not of CD16 (FcγRIII) and CD11b (CR3) in patients with localized and systemic bacterial infections. We found that during a peritonitis episode also changes in FcγR and CR expression on blood monocytes occurred, but not on neutrophils. The percentage CD16-positive monocytes and the CD32 MFI on monocytes did not increase. Maybe the differences between our results and those of Leino et al. can be explained by the fact that not all of our patients had a fever during peritonitis. However, systemic manifestations such as an increased total WBC count were present in 6 out of 9 children in the present study. Leino et al. showed that C-reactive protein level and leukocyte count was positively correlated with the level of FcγRII. We did not measure C-reactive protein in our patients. Therefore, we cannot exclude that FcγRII function may be suboptimal in our children. With regard to the monocyte population we expected that, if the CD32 function would have been normal in our PD patients, the expression would increase during an infection, as has been reported by Nakatini et al. [24]. However, this did not occur in our study. The discrepancy between our results and the fore mentioned might be caused by the uremic state, the toxicity of the dialysate of the PD children or the source of infection. Methodological differences such as the diversity of monoclonal antibodies which are used, might also influence the results.

The phenotype of peritoneal macrophages in adult PD patients with peritonitis has been studied cross sectionally by Hart et al. [25]. They showed an increased FcγR and a decreased CR expression on macrophages. However, the results were compared with monocytes from healthy persons and not with monocytes or macrophages from the same PD patients without peritonitis. We performed a follow-up study and found that the CD16, CD64 and CD35 but not the CD32 and CD11b MFI on macrophages increased during a peritonitis episode. Neutrophils in the peritoneal dialysis effluent had no increased MFI but the percentage receptor positive neutrophils increased, probably because of high influx from the blood circulation. Some studies demonstrated that gram-negative or gram-positive bacterial infections would result in different FcγR and CR alterations [26,27]. We could not establish a significant difference between gram-positive or gram-negative peritonitis, although this may have been attributable to the low number of patients. The children with a peritonitis incidence of < 1 episode per year had no different FcγR and CR expression, both in blood and dialysate, from children with an incidence of < 1 episode per year. Holmes et
FcyR and CR expression in PD

al. found a trend towards a higher FcyR and CR expression on peritoneal neutrophils and macrophages in CAPD patients with a history of a high peritonitis incidence compared to a low peritonitis incidence [28]. However, it seems that the increased peritonitis incidence in some patients cannot be explained by differences in FcyR or CR expression only. The peritonitis incidence in pediatric PD patients is higher than in adults [29-31]. We compared the results in children with those performed in adult PD patients, and found no differences in FcyR and CR expression levels between the groups. Because the number of adult PD patients was so low (n=4) we did not include the results in this study.

In conclusion, peritoneal neutrophils and macrophages of PD children showed lower percentages of FcyR and CR positive cells combined with an increased MFI, indicating a state of activation. During peritonitis, an upregulation of FcyR and CR expression levels could be demonstrated. However, not all cells and receptors could be upregulated maximally. This was especially found for the neutrophils and for FcyRII (CD32). The high turnover of cells into the peritoneal cavity, the uremic environment and the toxicity of the dialysate are probably responsible for this. The suboptimal receptor expression might be one of the determinants for the occurrence of infections.

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